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(54) Title: MODULATION OF APOPTOSIS

(57) Abstract: Methods and compositions for modulating apoptosis in cells and patients are provided. One method comprises selecting a compound which affects the formation of large channels comprised of multiple molecules of a pro-apoptotic member of the BCL-2 family, then administering the compound to the cell or patient. Another method comprises selecting a compound which affects the formation of a channel that includes at least four molecules of a pro-apoptotic BCL-2 family member polypeptide, then administering the compound to the cell or patient. Compounds which affect these channel characterictics are also provided. Additionally, methods for identifying apoptosis-modulating compounds using lipid membranes are provided. One method involves contacting a compound of interest with a lipid membrane which contains an ion-channel formed by an anti-apoptotic or pro-apoptotic polypeptide of the BCL-2 family and assaying for changes in the size of the pore. Changes in ion conductance properties of the channel, including ion selectivity, the ability to allow the passage of cytochrome c and/or FD10, single channel conductance and rectification are also useful characteristics for identifying apoptosis-modulating compounds.

MODULATION OF APOPTOSIS

Reference to Government Grant

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Cross-Reference to Related Applications

This application is a continuation-in-part of International Application PCT US99/17276, filed July 30, 1999, which is a continuation-in-part of U.S. application 09/127,048, filed on 31 July 1998, which claims priority to U.S. Provisional Application Serial No. 60/061,823, filed on 14 October 1997. These three applications are hereby incorporated by reference herein.

Background of the Invention

(1) Field of the Invention

This invention relates generally to the regulation of apoptosis and to compounds which modulate apoptosis, both antagonists and agonists, and more particularly, to methods for modulating apoptosis in cells and in animals, and to methods for identifying compounds with pro-apoptotic or anti-apoptotic activity.

(2) Description of the Related Art

Programmed cell death, also referred to herein as apoptosis, plays an indispensable role in the development and maintenance of homeostasis within all multicellular organisms (Raff, *Nature 356*:397-400, 1992). Genetic and molecular analysis from nematodes to humans indicates that the apoptotic pathway of cellular suicide is highly conserved (Hengartner and Horvitz, *Cell 76*:1107-1114, 1994). In addition to being essential for normal development and maintenance, apoptosis is important in the defense against viral infection and in preventing the emergence of cancer.

The BCL-2 family of proteins constitutes an intracellular checkpoint of apoptosis. The founding member of this family is the apoptosis-inhibiting protein encoded by the *bcl-2*

protooncogene which was initially isolated from a follicular lymphoma (Bakhshi et al., *Cell 41*:889-906, 1985; Tsujimoto et al, *Science 229*:1390-1393, 1985; Cleary and Sklar, *Proc Natl Acad Sci USA 82*:7439-7443, 1985). The Bcl-2 protein is a 25 kD, integral membrane protein localized to intracellular membranes including mitochondria. This factor extends survival in many different cell types by inhibiting apoptosis elicited by a variety of death-inducing stimuli (Korsmeyer, *Blood 80*:879-886, 1992).

The family of BCL-2-related proteins is comprised of both anti-apoptotic and pro-apoptotic members that function in a distal apoptotic pathway common to all multi-cellular organisms. It has been suggested that the ratio of anti-apoptotic (*e.g.*, Bcl-2, Bcl-x_L, Mcl-1 and A1) to pro-apoptotic (*e.g.*, Bax, Bak, Bcl-x_S, Bad, Bik and Bid) molecules may be involved in determining whether a cell will respond to a proximal apoptotic stimulus (Oltvai et al., *Cell 74*:609-619, 1992; Farrow, et al., *Curr. Opin. Gen. Dev. 6*: 45-49, 1996). Because members of this family can form both homodimers and heterodimers, the latter often between anti- and pro-apoptotic polypeptides, the balance of these homodimers and heterodimers could play a role in regulating apoptosis (Oltvai and Korsmeyer, *Cell 79*:189-192, 1994).

Members of the BCL-2 family are defined by sequence homology that is largely based upon conserved motifs termed BCL-Homology domains. (Yin et al, *Nature 369*:321-323, 1994). BCL-Homology domains 1 and 2 (BH1 and BH2) are important in dimerization and in modulating apoptosis (Yin et al., *supra*). A third homology region, BH3, is found in some family members and is important in dimerization as well as promoting apoptosis (Boyd et al., *Oncogene 11*:1921-1928; Chittenden et al., *Embo J 14*:5589-5596, 1995). BH4, the most recently identified homology domain, is present near the amino terminal end of some pro-apoptotic family members (Farrow et al., *supra*).

All known members of the BCL-2 family other than Bad and Bid have a C-terminal membrane-anchoring tail (TM). BCL-2 family members with a TM are intracellular integral membrane proteins most convincingly localized to mitochondria, the endoplasmic reticulum and the nuclear membrane. The intracellular membrane localization of BCL-2 family members together with the identification of structural similarity between the Bcl-x_L monomer and the ion channel forming toxins of colicin and diphtheria toxin B fragment (Muchmore et al., *Nature 381*:335-341, 1996) has prompted electrophysiological studies by several groups on the ability of BCL-2 family members to form ion channels in artificial lipid membranes.

These studies have reported that the anti-apoptotic family members Bcl-x_L and Bcl-2 lacking the TM (Bcl-$x_L\Delta$TM and Bcl-2ΔTM) insert into synthetic lipid vesicles at pH values below 5.5, but have little or no detectable ability to form ion channels when added to

lipid vesicles at pH values above 5.5 (Minn et al., *Nature 385*:353-356, 1997; Schendel et al., *PNAS USA* 94:5113, 1997; and Antonsson et al., *Science 277*:370-372, 1997). When added to planar lipid bilayers at physiological pH, Bcl-$x_L\Delta$TM forms channels which exhibit multiple conductance states and which have an ion selectivity sequence of $K^+=Na^+>Ca^{2+}>Cl^-$ (Minn et al., *supra*). Bcl-2ΔTM also forms a channel in lipid bilayers at physiological pH having multiple channel conductance states, with a primary conductance of 18 ± 2 pS being consistent with channel formation by Bcl-2 homodimers (Schendel et al., *supra*). Schendel et al reported that the Bcl-2ΔTM channel is cation selective at pH 5.4 but did not test ion selectivity at neutral pH.

It has also been reported that BaxΔTM has an intrinsic ability to form ion channels in liposomes and that Bcl-2 antagonizes this effect at physiological pH (Antonsson et al., *supra*). This reference also reported that BaxΔTM forms voltage-dependent channels in planar lipid bilayers at pH 7.0 that are slightly cation selective, with a permeability ratio of Na^+ to Cl^- of about 2.1.

None of these electrophysiological studies have shown any functional connection between the ion channel forming ability of BCL-2 family members and apoptosis. Antonsson et al. speculated that the differences between the intrinsic channel forming properties of Bax and Bcl-x or Bcl-2 may be related to the pro-apoptotic and anti-apoptotic functions of these substances, however, what the connection might be between ion channel activity and apoptosis was not disclosed or suggested in this reference. Schendel et al. suggested that Bcl-2 may allow transport of an ion or protein across membranes in a direction that is cytoprotective whereas Bax does the opposite, however, this reference indicated that what Bcl-2 is intended to transport across membranes remains unanswered. Minn et al. speculated that although Bcl-x_L forms an ion-selective channel , it is also possible that Bcl-x_L regulates the passage of proteins and cytochrome c was mentioned as a protein that resides in the mitochondrial inter membrane space and its redistribution was noted by this reference to have been suggested to promote apoptosis. Nevertheless, this group provided further speculation that the ability of Bcl-x_L to promote cell survival may not result solely from the ability to form a channel so that no clear mechanism connecting the channel forming ability of Bcl-x_L and apoptosis was suggested by this reference. Furthermore Minn et al. provided no experimental results suggesting what the connection between ion channel formation and apoptosis might be.

Other groups have provided evidence suggesting that cytochrome c may be involved in apoptosis. The release of cytochrome c from mitochondria has been shown to be a regulatory event in Bax-induced apoptosis (Kluck et al., *Science 275*:1132-1136, 1997;

Shimizu et al., *Nature 399*:483-487, 1999). Nevertheless, none of these studies provided any suggestion that BCL-2 family members, acting alone, are responsible for cytochrome c release.

Some disease conditions are believed to be related to the development of a defective down-regulation of apoptosis in the affected cells. For example, neoplasias may result, at least in part, from an apoptosis-resistant state in which cell proliferation signals inappropriately exceed cell death signals. Furthermore, some DNA viruses such as Epstein-Barr virus, African swine fever virus, and adenovirus, parasitize the host cellular machinery to drive their own replication and at the same time modulate apoptosis to repress cell death and allow the target cell to reproduce the virus. Moreover, certain disease conditions such as lymphoproliferative conditions, cancer including drug resistant cancer, arthritis, Crohn's disease, inflammation, autoimmune diseases and the like may result from a down regulation of cell death regulation. In such disease conditions it would be desirable to promote apoptotic mechanisms.

Conversely, in other disease conditions it would be desirable to inhibit apoptosis such as in the treatment of immunodeficiency diseases, including AIDS, senescence, neurodegenerative disease, ischemic and reperfusion cell death, infertility, wound-healing, and the like. In the treatment of such diseases it would be desirable to inhibit apoptotic mechanisms.

Thus, it would be desirable to elucidate the biochemical mechanisms involved in the regulation of apoptosis by BCL-2 family members and to utilize these mechanisms as a basis for identifying compounds which promote or inhibit death. Such compounds would be useful in developing treatment regimens for advantageously modulating the apoptotic process in disease conditions involving either inappropriate repression or inappropriate enhancement of cell death.

Summary of the Invention

Accordingly, the inventors herein have succeeded in discovering a novel method for identifying apoptosis modulating compounds, the method comprising contacting a lipid membrane with a pore-forming pro-apoptotic member of the BCL-2 family and a compound to be tested for modulation of apoptotic activity; and determining whether the compound regulates the formation of large pores in the lipid membrane, where an increase in the number of large pores indicates that the compound is a promoter of apoptotic activity and a decrease in the number of large pores indicates that the compound is an inhibitor of apoptotic activity.

The inventors have also discovered a novel method of inducing apoptosis in a cell comprising: selecting a compound which induces the formation of a pore of approximately 20

- 35 Å in a lipid membrane, wherein said selecting comprises testing the compound for the induction of said pore formation; and administering the compound to the cell.

The inventors have also discovered a novel method of inhibiting apoptosis in a cell comprising: selecting a compound which inhibits the formation of a pore of approximately 20 - 35 Å in a lipid membrane, wherein said selecting comprises testing the compound for the inhibition of said pore formation; and administering the compound to the cell.

The inventors have also discovered a novel method of treating a patient having a condition mediated by excessive down-regulation of apoptosis comprising: selecting a compound which induces the formation of a pore of approximately 20 - 35 Å, wherein said selecting comprises testing the compound for the induction of said pore formation; and administering the compound to the patient.

The inventors have also discovered a novel method of treating a patient having a condition mediated by excessive apoptosis comprising: selecting a compound which inhibits the formation of a pore of approximately 20 - 35 Å, wherein said selecting comprises testing the compound for the inhibition of said pore formation; and administering the compound to the patient.

The inventors have also discovered novel compounds that promote the formation of large pores in lipid membranes, and novel compounds that inhibit the formation of large pores in lipid membranes.

Among the several advantages found to be achieved by the present invention, therefore, may be noted the provision of methods for modulating apoptosis in cells, methods for modulating apoptosis in patients having a condition which is mediated by excessive apoptosis or excessive down-regulation of apoptosis; the provision of methods for screening compounds for apoptosis-modulating activity that allow compounds to be evaluated without regard to their ability to penetrate into cells or stability in a cell-based assay; the provision of methods that can be used to rapidly screen large numbers of compounds for apoptosis-modulating activity; and the provision of death agonists and antagonists identified by these lipid-bilayer screening methods.

Brief Description of the Drawings

Figure 1 illustrates the effect of pH on Bax and Bcl-2 induced release of Cl^- ions from synthetic lipid vesicles, showing (FIG. 1A) vesicles incubated with BaxΔTM at pH 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0, (FIG. 1B) vesicles incubated with Bcl-2ΔTM at pH 3.5, 4.0, 4.5 and 5.0, (FIG. 1C) vesicles incubated with BaxΔTM at pH 6.0 shifted to pH 4.0 at the time point indicated with the vertical arrow, and (FIG. 1D) vesicles incubated with Bcl-2ΔTM at pH 5.0 shifted to pH 4.0 at the time point indicated with the vertical arrow;

Figure 2 illustrates the low pH formation of Bax channels in planar lipid bilayers as characterized by the currents appearing after addition of BaxΔTM to the cis chamber of an established planar lipid bilayer in a 450/150 mM cis to trans KCl gradient at pH 4.0, showing (FIG. 2A) a tracing of an initial, inward Cl- current that appeared spontaneously at 0 volts (O_S), (FIG. 2B) tracings of the currents that occurred at +40 mV (upper tracing) or -40 mV (lower tracing) after removal of soluble BaxΔTM from the cis chamber showing the transition from the O_S to a large open pore with * denoting a direct C-O_S to O_2 transition, and (FIG. 2C and 2D) current-voltage (I/V) plots for the mature pore at pH 4.0 (FIG. 2C) and following a shift to pH 7.0 in the presence of the 450/150 mM KCl gradient (open circle) or in symmetric 150 mM KCl (filled square) (FIG. 2D);

Figure 3 illustrates the incorporation of Bax into planar lipid bilayer membranes following addition of BaxΔTM-containing unilamellar proteoliposomes to the cis chamber of an established lipid bilayer, showing (FIG. 3A) a tracing of an initial inward Cl^--selective current in the presence of a 450/150 mM KCl gradient, (FIG. 3B) tracings of the current of a single Bax channel in symmetrical 150 mM KCl at different voltages, (FIG. 3C) an I/V plot of the Bax channel, and (FIG. 3D) longer tracings of the current which demonstrate rectification at 70 mV (upper tracing) and closures at -70 mv (lower tracing);

Figure 4 illustrates the low pH formation of Bcl-2 channels in planar lipid bilayers as characterized by the currents appearing after addition of Bcl-2ΔTM to the cis chamber of an established planar lipid bilayer in a 450/150 mM cis to trans KCl gradient at pH 4.0, showing (FIG. 4A) a tracing of an initial, outward K^+ current that appeared spontaneously at 0 volts (O_S), (FIG. 4B) channel transitions between the open (O_S) and closed (c) states at 0 volts for three duplicate experiments, (FIG. 4C) a histogram of the distribution of current amplitudes for the channel over 120 seconds, (FIG. 4D) an I/V plot of the Bcl-2 channel from (B), and (FIG. 4E) an I/V plot of the activity of the large pore that formed over time at pH 4.0 (closed square) and following a shift to pH 7.0 (open square) or in symmetric 150 mM KCl (filled square);

Figure 5 illustrates the incorporation of Bcl-2 into planar lipid bilayer membranes following addition of Bcl-2ΔTM-containing unilamellar proteoliposomes to the cis chamber of an established lipid bilayer, showing (FIG. 5A) a tracing of the initial K^+ current of two Bcl-2 channels that simultaneously appeared in the presence of a 450/150 mM KCl gradient, (FIG. 5B) tracings of the channel current of an established single Bcl-2 channel (upper tracing) resulting from application of a series of voltage steps (lower tracing) to the channel in a 450/150 mM KCl gradient, (FIG. 5C) tracings of a single Bcl-2 channel in symmetrical 150 mM KCl at different voltages, and (FIG. 5D) an I/V plot of the Bcl-2 channel;

Figure 6 illustrates models of the three-dimensional structure of the positively charged surface of the α-5 and α-6 helices of Bax (left side) and the negatively charged surface of the same region of Bcl-2 (right side) showing darker surfaces for the most highly charged regions, with linear interpolation for values in-between;

Figure 7 illustrates the aligned sequences of α-5 and α-6 helices in two anti-apoptotic (Bcl-x_L and Bcl-2) (SEQ ID NOS:2-3) and in two pro-apoptotic molecules (Bax and Bak) (SEQ ID NOS:4-5);

Figure 8 illustrates the effect of a death-promoting 11-mer Bax peptide on the conductance characteristics of a Bcl-2 channel in a planar lipid bilayer showing the conductance patterns of Bcl-2 (FIGS. 8A-D) and Bax (FIG. 8E) channels at 80 mV under voltage clamp conditions with the frequency that each channel is present at one of several conductance levels plotted against the conductance in picoamps in the absence (FIGS. 8A and 8E) or presence of the 11-mer peptide at 20 μM (FIG. 8B), 50 μM (FIG. 8C), or 100 μM (FIG. 8D);

Figure 9 illustrates the effect of the Bax death agonist peptide on the conductance and ion selectivity of a Bcl-2 channel showing an IV plot of channel activity at 150 mM symmetrical KCl and the pK/pCl calculated from the reversal potential in a 450:150 mM cis:trans KCl gradient for (FIG. 9A) a Bcl-2 channel, (FIG. 9B) a Bcl-2 channel after the addition of the Bax death agonist peptide to the cis side of the bilayer, and (FIG. 9C) a Bax channel, with the slope of the conductance at negative potentials indicated;

Figure 10 illustrates the members of the BCL-2 family showing the presence and location of homology domains BH1, BH2, BH3 and BH4, as well as the transmembrane domain (TM);

Figure 11 illustrates the aligned sequences of mouse and human Bax (SEQ ID NOS:6-7) and human and mouse Bcl-2 (SEQ ID NOS:8-9), with identical residues and conservative substitutions shaded dark and light, respectively, exon boundaries indicated by vertical dashed lines, and the BH1 and BH2 domains boxed;

Figure 12 illustrates the concentration dependence of carboxyfluorescein dequenching and pore sizing by dextran inhibition, where (A) shows the time dependence of fluorescence dequenching at selected BAX concentrations, • 1.82 nM, x 9.09 nM, Δ18.2 nM, and ■ 36.4 nM, and where the time constants, τ, are listed above the plot. Figure 12(B) shows a Hill plot of the velocity of dequenching with increasing BAX concentrations, where the slope of this plot at 0.2Vmax is 2.0±0.19 rising to 3.9±0.24 at $0.8V_{max}$, and the inset indicates the continuous slope across the studied BAX concentrations. Figure 12(C) shows inhibition of carboxyfluorescein dequenching at 9 nM BAX by 20 μM dextrans of 6, 9 and 39.4 kDa. The dequenching time constant, $\tau_{blocked}$, reflects the size-specific dextran block of carboxyfluorescein release (± standard deviation), and the exponential phase of dequenching is presented after removal of the linear release [equation (1)]. Figure 12 (D) shows a comparison of functional pore diameter determined by size-dependent dextran inhibition of dequenching at three BAX concentrations; 5 nM (∇), 9 nM (□) and 20 nM (Δ), and where time constants are averaged (n=3) and plotted with standard deviation at each dextran size. At each BAX concentration size-dependent dextran inhibition was fitted to a Gaussian peak and the peak center ± 1 standard deviation was determined (5 nM: 10.7±3.2 Å, 9 nM: 22.1±3.1 Å, and 21.4±5.2 Å). The vertical hatched column for each BAX concentration reflects the fitted peak with a width of two standard deviations;

Figure 13 illustrates BAX dependent dequenching from FITC-Dextran containing liposomes, where (A) shows that increasing fluorescence resulted upon release of 10kD fluorescein isothiocyanate-labeled dextran (FD10) from the vesicles as predicted by the Stern-Volmer analysis of concentration dependence of FITC-dextran fluorescence; in (B) the time constants for 0,10, 30 and 40 μM blocking dextran are plotted and fitted to a sigmoid curve and the IC50 for 9 kDa dextran block of FITC-dextran release was determined to be $4.05\pm0.46\times10^{-5}$; and

Figure 14 shows the characterization of BAX-mediated cytochrome c release, where (A) shows a comparison of the time course of dequenching for carboxyfluorescein (1.82 nM renatured BAX) and FCC containing vesicles (322 nM renatured BAX). The time constants, τ, for dequenching were 77.1±0.7 and 5.01±2.7 seconds respectively. In (B), the effect of extravesicular unlabelled cytochrome c on BAX-dependent FCC release was determined using 322 nM BAX. Time constants for dequenching were determined by fitting to equation 1 and are shown plotted on a logarithmic scale in the inset panel. This plot was fitted to a sigmoid function yielding an 1C50=9.05±0.2x104. For Figure 14(C), the time constant for dequenching of FCC was determined at 72 to 540 nM BAX and used to construct a Hill plot

from 0.95 to 0.05V_{max}. A linear plot with a slope of 4 is presented along with the standard error (n=3) for each experimental point. For Figure 14(D), the size—specific dextran block, $\tau_{blocked,}$ of BAX mediated FCC dequenching was determined to be 28.9±6.0 Å and dextrans with a Stokes diameter greater than this show decreasing inhibition.

Description of the Preferred Embodiments

The present invention is based on the surprising discovery that pro-apoptotic BCL-2 family members form pores that can regulate cytochrome c and/or 10kD fluorescein isothiocyanate-labeled dextran (FD10) passage out of mitochondria, and that the ability of cytochrome c and FD10 to pass through channels formed by these pro-apoptotic BCL-2 family members in lipid membranes is dependent upon whether the pro-apoptotic BCL-2 family members form a large pore in the membrane. Specifically, it has been found that two molecules of a pro-apoptotic BCL-2 family member (and, in particular BAX) can form membrane pores of approximately 10 Å, which do not allow cytochrome c to pass through, whereas at least four molecules of these same pro-apoptotic BCL-2 family members can form larger channels of approximately 20 - 22 Å in size, preferably approximately 20 - 29 Å in size, and more preferably approximately 20 - 35 Å in size that permit the passage of cytochrome c and/or FD10. When it is said that at least four molecules of a pro-apoptotic BCL-2 family member form a pore, it is meant to include pores that are formed with four such molecules, and pores that are formed with five, six or more of such molecules.

Furthermore, it has been discovered that BAX can act alone to form either the small channels (two Bax molecules form channels of about 10 Å) or the large channels (at least four Bax molecules form channels of about 20 - 35 Å), but that only the large channels transmit cytochrome c and/or FD10.

Furthermore, it has been discovered that compounds that act to regulate the formation of the large pores are also associated with the regulation of apoptosis. Any compound that regulated the formation of a large pore would be expected to have a regulatory affect upon apoptosis. For example, one member of the pro-apoptotic BCL-2 family can act together with another pro-apoptotic member of that family to form large pores having four pro-apoptotic molecules therein, and such large pores can transmit cytochrome c. Also, compounds that are anti-apoptotic members of the BCL-2 family may also act to regulate the formation of the large pores.

Furthermore, it has been discovered that the ion-selectivity of channels formed by BCL-2 family members in lipid membranes is dependent upon whether the polypeptide has

anti-apoptotic activity or pro-apoptotic activity, specifically, anti-apoptotic polypeptides are K^+-selective while pro-apoptotic polypeptides are Cl^--selective. Moreover, it has been discovered that a compound which modulates the above selectivities can modulate apoptosis. These discoveries were unexpected because there was no previous indication that the channels made by pro-apoptotic family members are large enough to allow passage of cytochrome c, and because channels formed by Bax, which is pro-apoptotic, had previously been reported to be cation selective at pH 7.0 with a permeability ratio of Na^+ to Cl^- of about 2.1 (Antonsson et al., *supra*), whereas it is now herein reported that Bax channels are anion selective. Also, no connection had previously been made between the ability of a channel to allow cytochrome c to pass, or the ion selectivity of a channel formed by a BCL-2 family member, and apoptotic activity.

The term "ion selective" is used herein to mean a characteristic of a membrane channel or pore where a particular ion is favored for passage through the channel over another ion. For example, "K^+ selective" refers to a channel which favors passage of K^+ over another ion, for example Cl^-. The degree of ion selectivity between two ions can be expressed as a ratio, also known as a permeability ratio. For example, a channel which has a permeability ratio of Na^+ to Cl^- of 2.1 allows 2.1 times as much Na^+ as Cl^- to pass though over a given time period and can be designated Na^+ selective or cation selective.

As used herein, the terms "large pore" or "large channel" refer to pores or channels in a lipid membrane of about 20 - 22 Å, preferably about 20 - 29 Å, and more preferably about 20 - 35 Å in size that are formed by at least four molecules of a pro-apoptotic BCL-2 family member. The terms "small pore" or "small channel" refer to pores or channels in a lipid membrane of about 10 - 11 Å size that are formed by no more than two molecules of a pro-apoptotic BCL-2 family member.

Therefore, the present invention provides novel methods for modulating apoptosis which involves administering to cells compounds which regulate the formation of large pores. If an increase in apoptotic activity is desired, the cells are administered a compound which promotes the formation of a large pore. (*i.e.*, a cytochrome c and/or FD10 transporting or releasing pore of at least four molecules of a pro-apoptotic BCL-2 family member (for example, Bax, Bad, Bak, Bik, or Bid)); if an decrease in apoptotic activity is desired, the cells are administered a compound which inhibits the formation of a large pore.

As used herein, the term "compound" is intended to include inorganic or organic chemical compounds as well as biochemical molecules such as nucleic acids, proteins, lipids, lipoproteins, and carbohydrates.

In preferred embodiments, when apoptosis is to be induced in a cell, the cell is administered Bax, or more preferably, an 11-mer polypeptide with the sequence ECLKRIGDELD, presented herein as SEQ ID NO:1. This compound is a fragment of the BH3 domain of Bax corresponding to amino acids 61-71 of full length Bax (SEQ ID NO:7). As demonstrated in Examples 8 and 9, this compound, when added to a membrane comprising a anti-apoptotic BCL-2 family member, changes the ion selectivity of the pore formed by the anti-apoptotic BCL-2 family member from Cl^- selective to K^+ selective. Analogous polypeptides from other pro-apoptotic BCL-2 family members are also encompassed within these embodiments. As used herein, the term "polypeptide" encompasses a linear sequence of two or more amino acids, linked by peptide bonds.

When it is desired to promote apoptosis, it is also preferred that a sufficient amount of the polypeptide having the sequence ECLKRIGDELD (SEQ ID NO:1) is administered to form large pores in mitochondrial membranes.

In other preferred embodiments, when apoptosis is to be inhibited in a cell, the cell is administered an 11-mer polypeptide with the sequence LTLRQAGDDFS, presented herein as SEQ ID NO:2. This compound is a fragment of the BH3 domain of Bcl-2 corresponding to amino acids 95-105 of full length Bcl-2 (SEQ ID NO:9). As demonstrated in Example 9, this compound, when added to a membrane comprising a pro-apoptotic BCL-2 family member, changes the ion selectivity of the pore formed by the pro-apoptotic BCL-2 family member from K^+ selective to Cl^- selective. Analogous polypeptides from other anti-apoptotic BCL-2 family members are also encompassed by these embodiments.

In other preferred embodiments, a polypeptide or polypeptide derivative is administered to a cell, wherein the polypeptide comprises SEQ ID NO:1 or SEQ ID NO:2. In these embodiments, the polypeptide can be any length, including a full length BCL-2 family member. As used herein, the term "administer" includes indirect administration of the compound, for example, treatment with a nucleotide sequence which encodes the polypeptide compound operably linked to a promoter which allows the expression of the polypeptide.

As used herein, a polypeptide derivative includes any functional variant or conservative substitution of the polypeptide. A functional variant of a naturally-occurring polypeptide contains one or more amino acid substitutions in that sequence which do not destroy the ability of the resulting polypeptide to function to modulate apoptosis. Preferably, amino acid substitutions in functional variants are conservative amino acid substitutions, which refer to the interchangeability of residues having similar side chains. Conservatively substituted amino acids can be grouped according to the chemical properties of their side chains. For example, one grouping of amino acids includes those amino acids have neutral

and hydrophobic side chains (A, V, L, I, P, W, F, and M); another grouping is those amino acids having neutral and polar side chains (G, S, T, Y, C, N, and Q); another grouping is those amino acids having basic side chains (K, R, and H); another grouping is those amino acids having acidic side chains (D and E); another grouping is those amino acids having aliphatic side chains (G, A, V, L, and I); another grouping is those amino acids having aliphatic-hydroxyl side chains (S and T); another grouping is those amino acids having amine-containing side chains (N, Q, K, R, and H); another grouping is those amino acids having aromatic side chains (F, Y, and W); and another grouping is those amino acids having sulfur-containing side chains (C and M). Preferred conservative amino acid substitutions groups are: R-K; E-D, Y-F, L-M; V-I, and Q-H.

A functional variant as used herein can also include modified sequences in which one or more amino acids have been inserted, deleted, or replaced with a different amino acid or a modified amino acid or unusual amino acid, as well as modifications such as glycosylation or phosphorylation so long as the polypeptide containing the modified sequence retains the ability to modulate apoptosis. By retaining the biological activity, it is meant that the modified polypeptide can function to modulate apoptosis..

Because of the universality of the apoptotic mechanisms which are the subject of the present invention, it is believed that apoptotic mechanisms of any cell of any multicellular organism would be affected by the methods presented herein. Included are cells from any invertebrate or vertebrate, including humans. Preferred are cells from a patient with a deleterious condition which is mediated by impaired regulation of apoptosis - either excessive apoptosis or excessive down-regulation of apoptosis. The term "patient" is used herein to include any vertebrate. Most preferred are cells from a human patient which has a condition mediated by impaired regulation of apoptosis. Examples of conditions which may be mediated by excessive down-regulation of apoptosis are neoplasias, diseases caused by Epstein-Barr virus, African swine fever virus, and adenovirus, lymphoproliferative conditions, cancer, arthritis, Crohn's disease, inflammation, and autoimmune disease. Examples of conditions which may be mediated by excessive apoptosis are immunodeficiency diseases, senescence, neurodegenerative disease, ischemic and reperfusion cell death, infertility, and conditions characterized by slow wound healing.

Compositions comprising the compound which modulates apoptosis can be administered to a cell *ex vivo*. For example, if a patient has a metastatic cancer which is mediated by down-regulation of apoptosis, the patient can be treated by removing bone marrow cells, treating the patient with a sublethal dose of radiation to kill the cancer in the patient, treating the bone marrow cells with a pro-apoptotic compound of the present

invention to kill the cancer cells in the bone marrow, then transplanting the bone marrow back into the patient.

In embodiments where it is desirable to administer the compound to cells *ex vivo*, for example -- *in vitro* -- the methods for introduction and administration of small peptides to cultured cells are well developed with a variety of methods available. Lauer, J. L., and G. B. Fields, in *Methods in Enzymology, 289:*564 - 571 (1997).

One such method involves the application of a controlled electrical field to cultures of cells, which causes the formation of large pores in the cellular membranes so that if the maneuver is done in the presence of a peptide and in a buffer that is not toxic to the cells, both the buffer and the peptide enter the cytoplasm of the cell. Subsequently when the electrical field is turned off the membrane pores close and the peptide is present in the cellular cytoplasm. Schieffer *et al., J. Biol Chem., 271(17)*: 10329 - 10333 (1996), show an example of the introduction of an antibody (150 kDa) into a cell.

Alternatively, large reversible membrane pores can be generated using bacterial toxins as shown in Damaj *et al., J. Biol. Chem., 271(22):*12783 - 12789 (1996), and used to introduce antibodies or inhibiting peptides into cells to block the intracellular release of calcium after IL-8 binding to cell surface receptors. The pores reported by Damaj *et al.* were created using 0.01% saponin which was washed away in Hanks Buffered Saline Solution allowing the membranes to re-seal. Subsequently the cells were studied for an increase in intracellular calcium after IL-8 application, which demonstrated that membranes had resealed, the peptides were inside the cells and that the cells were biologically responsive. Other toxins, including the streptolysin O are commonly used to administer many molecules to the interior of cultured cells. In addition to biological products synthetic compounds have been used to permeabilize the cell and can be obtained as commercial kits for the introduction of 2-3 kDa peptides (*Id.*, Lauer & Fields, 1997).

The peptide to be administered can also be prepared with a short leading sequence that has membrane translocation activity as done in Lin *et al., J. Biol. Chem., 27024):*14255 - 14258 (1995). In many cells this introduces an active peptide into the cellular cytoplasm without compromising the cellular plasma membrane. For this reason such an approach can be desirable if the modified peptide is active. A variety of biologically active membrane transport signals are available for use in this method including eukaryotic protein transport signal sequences and insect antennapedia sequences. In a modification of this technique a ligand or an antibody to a cell surface receptor can be added to a cationic lipid complex of the peptide to promote cell specific and efficient receptor mediated endocytosis. The inclusion of a fusogenic viral protein destabilizes the endosome and releases the peptide

to the cellular cytoplasm as shown by Simoes *et al., Gene Therapy, 5*: 955 - 964 (1998). Although tested in this work for gene administration, this would be expected to be applicable to peptides also.

Another method that applies a low stress to the target cells is the administration of peptides encapsulated in artificial liposomes (*Id.,* Lauer and Fields (1997)), and by Amselem *et al., Chem. Phys. Lipid, 64*: 219 - 237 (1993).

Finally the most direct approach for direct *in vivo* administration of peptides to cells is the micro-injection using glass pipettes of the peptide one cell at a time. Recent work has shown that the direct injection of caspase 8 will promote apoptsis (Srinivasan *et al., J. Biol. Chem., 273(8)*: 4523 - 4529 (1998), so that this method can be directly applied to the study of peptides modulating apoptosis in cells.

Preferably, however, the compounds of the present invention are administered to cells which are within a living patient. The invention compounds can be administered by any suitable route known in the art including, for example, intravenous, subcutaneous, intramuscular, transdermal, intrathecal, or intracerebral. The compositions can also be administered to target cells directly in *ex vivo* treatment protocols. Administration can be either rapid as by injection or over a period of time as by slow infusion or administration of a slow release formulation. For treating cells in the central nervous system, administration can be by injection into the cerebrospinal fluid.

In embodiments in which it is desirable to administer polypeptides to cells or to a patient, these can be administered by administering a polynucleotide encoding the polypeptide to the cell or patient.

It is contemplated that the compounds of the present invention are usually administered as compositions in the form of a pharmaceutical preparations. Such preparations are made in a manner well known in the pharmaceutical art. One preferred preparation utilizes a vehicle of physiological saline solution, but it is contemplated that other pharmaceutically acceptable carriers such as physiological concentrations of other non-toxic salts, five percent aqueous glucose solution, sterile water or the like may also be used. It may also be desirable that a suitable buffer be present in the composition. Such solutions can, if desired, be lyophilized and stored in a sterile ampoule ready for reconstitution by the addition of sterile water for ready injection. The primary solvent can be aqueous or alternatively non-aqueous.

The carrier can also contain other pharmaceutically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier may contain still other

pharmaceutically-acceptable excipients for modifying or maintaining release or absorption or penetration across the blood-brain barrier. Such excipients are those substances usually and customarily employed to formulate dosages for parenteral administration in either unit dosage or multi-dose form or for direct infusion by continuous or periodic infusion.

It is also contemplated that certain formulations comprising the agent are to be administered orally. Such formulations are preferably encapsulated and formulated with suitable carriers in solid dosage forms. Some examples of suitable carriers, excipients, and diluents include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, gelatin, syrup, methyl cellulose, methyl- and propylhydroxybenzoates, talc, magnesium, stearate, water, mineral oil, and the like. The formulations can additionally include lubricating agents, wetting agents, emulsifying and suspending agents, preserving agents, sweetening agents or flavoring agents. The compositions may be formulated so as to provide rapid, sustained, or delayed release of the active ingredients after administration to the patient by employing procedures well known in the art. The formulations can also contain substances that diminish proteolytic degradation and/or substances which promote absorption such as, for example, surface active agents. Alternatively, the carrier can be a gelatin capsule, or coated tablet, or the like which is suitable for administering a unit dosage to a patient.

The agent is administered to the organism in an amount effective to modulate apoptosis of target cells within the organism. The specific dose is calculated according to the approximate body weight or body surface area of the patient or the volume of body space to be occupied. The dose will also be calculated dependent upon the particular route of administration selected. Further refinement of the calculations necessary to determine the appropriate dosage for treatment is routinely made by those of ordinary skill in the art. Such calculations can be made without undue experimentation by one skilled in the art in light of the activity disclosed herein in apoptosis assays. Exact dosages are determined in conjunction with standard dose-response studies. It will be understood that the amount of the composition actually administered will be determined by a practitioner, in the light of the relevant circumstances including the condition or conditions to be treated, the choice of composition to be administered, the age, weight, and response of the individual patient, the severity of the patient's symptoms, and the chosen route of administration. Dose administration can be repeated depending upon the pharmacokinetic parameters of the dosage formulation and the route of administration used.

In some embodiments of the present invention, the compound which is used to mediate apoptosis in a cell is first selected wherein the selecting comprises testing the

compound for the ability to either (a) affect the ability of a channel formed by a BCL-2 family member to transport cytochrome c out of the mitochondria, or (b) affect the ion selectivity of such a channel. This selecting step is intended to apply to all compounds which are known or not known to mediate apoptosis, and comprises, in whole or in part, testing for the ability or inability of a particular compound to mediate apoptosis. Such testing can involve either determining or confirming previously suspected channel forming activity. The process of testing and selecting a compound which mediates apoptosis is also intended as an additional embodiment of the invention comprising methods for identifying apoptosis-modulating compounds.

Based on the structural features of the critical amino acid sequence of the peptides of the present invention that permit either formation of a pore for cytochrome c and/or FD10 passage or binding to a BCL-2 family member to form the desired pore, one can develop non-peptide derivatives that are capable of such action.

The techniques for development of such peptide mimetics are well known in the art (see, for example, Navia et al., *Trends Pharm. Sci. 14*:189-195, 1993; Olson et al., *J. Med. Chem. 36*:3039-3049, which are herein incorporated by reference). Typically this involves identification and characterization of the protein target as well as the protein ligand using X-ray crystallography and nuclear magnetic resonance technology such as has been done with BCL-2 family members (see, *e.g.*, Figure 6). Using information learned from the structure of the target protein and ligand, a pharmacophore hypothesis is developed and compounds are made and tested in an assay system.

Thus, in some embodiments of the invention, apoptosis-modulating compounds are conveniently selected or identified using methods which involve assaying the effect of candidate compounds on the ion selectivity of a channel in a lipid membrane, the channel being comprised of a polypeptide from the BCL-2 family. As used herein, the terms "lipid membrane" is meant to include liposomes and proteoliposomes, as well as lipid bilayers.

The lipid membrane assay described herein has several advantages over a whole cell assay to identify candidate death agonists and antagonists. First, it provides specific information about the biochemical effect of compounds on what is believed to be an underlying biochemical mechanism of the regulation of apoptosis by BCL-2 family members. In addition, a lipid membrane assay will identify more candidate death agonists and antagonists than the whole cell assay. For example, some compounds that have apoptotic activity are not detected by the whole cell assay due to cell uptake problems or degradation of the compound by the cell. However, such problems might be overcome in further development or modification of a lead compound identified by a lipid membrane assay.

When a lipid bilayer is used in the method, the lipid bilayer can comprise a double layer of lipid in a hydrophilic environment. In some embodiments, the lipid bilayer comprises a planar bilayer, while in other embodiments, the lipid membrane comprises a proteoliposome. Preparation of such lipid bilayers can be accomplished using biochemicals and techniques typically used in bilayer reconstitution experiments. See, *e.g.*, Hanke, W. and Schlue, W.-R., *Planar Lipid Bilayers*, D.B. Sattelle, ed., Academic Press, 1993. The lipids in the bilayer can comprise natural lipids purified from biological membranes and/or synthetic lipids. Examples of natural lipids include but are not limited to Azolectin, a commercially available lipid extract from soybeans (Sigma, St. Louis, MO), and phosphatidylcholine and phosphatidylethanolamine purified from egg yolk. Synthetic lipids can be lipids which are pure in their head-group and hydrocarbon composition or lipids that are not normally present in biological membranes. Preferably, the lipid bilayer comprises at least 10% negatively charged lipids. The lipid composition of planar bilayers preferably comprises about 10-20% choline and 80-90% negatively charged lipids. Liposomes preferably comprise about 60% 1,2 dioleoyl phophatidyl choline and about 40% 1,2 dioleoyl phosphatidyl glycerol.

A planar lipid bilayer is established using standard techniques (Hanke and Schlue, *supra*). In brief, a double compartment chamber with an aperture having a diameter of about 0.1 to about 1 mm and a thickness of about 1 mm or less between the compartments is filled with an aqueous solution to completely cover the aperture. The aqueous solution is typically a KCl solution although other salt solutions can be used, including NaCl and $CaCl_2$. Preferably, the salt solution is comprised of monovalent cations and monovalent anions. A drop of lipid dissolved in an organic nonpolar solvent is then spread across the aperture and allowed to thin to the thickness of a lipid bilayer. The concentration of lipids in the lipid solution is between about 1 and 100 mg/ml, preferably between 20 and 80 mg/ml, and more preferably between 30 and 70 mg/ml. Most preferably, the lipid concentration is 50 mg/ml. Decane is generally used as the solvent, but the solvent can also be other short chain alkanes such as hexane as well as long-chain alkanes and alkenes such as squalene. When the solvent is decane, the lipid bilayer will have a specific capacity of about 0.4 $\mu F/cm_2$. Preferably, the aperture is pretreated with lipid by covering it with the lipid solution and then evaporating the solvent. After pretreatment, the compartments are filled with the aqueous solution and a second drop of lipid solution is spread on the aperture for forming the lipid bilayer.

The planar lipid bilayer can also be prepared by other techniques known in the art such as by forming a folded bilayer across the aperture from two monolayers, one in each compartment, and apposition of two monolayers at the tip of a patch pipet (Hanke and Schlue, *supra*).

The lipid membrane comprises at least one channel comprised of an anti-apoptotic or pro-apoptotic polypeptide from the BCL-2 family (Fig. 10). The sequences of these polypeptides are readily available on GenBank and in the scientific literature. The BCL-2 related polypeptide can be a conservatively substituted variant of the naturally-occurring sequence, or a fragment thereof.

Fragments of BCL-2 related polypeptides useful in the invention are preferably those having sufficient length to form a channel in a lipid membrane. Construction of such fragments and testing them for channel forming activity or for alteration of channels provided by a BCL-2 family member, either endogenously present or administered with the fragment, can be done using well-known deletion-mutagenesis procedures and the procedures described herein. Such fragments of BCL-2 related polypeptides can, in some embodiments, include polypeptides having a length up to and including the full length BCL-2 family member.

The channel can also be formed by any subsequently discovered BCL-2 family member that has channel forming activity *in vitro* and has anti-apoptotic or pro-apoptotic activity. It is preferred that the BCL-2 family member is one that includes a BH3 domain.

For the formation of pores of sufficient size to allow passage of cytochrome c and/or FD10, renaturation of purified bacterially expressed Bax should be in 8M urea with gradual refolding by dialysis. See, *e.g.* Anfinsen *et al.*, *Adv. Protein Chem. 29*:205-300, 1975.

Preferably, the polypeptide used to form the channel lacks a C-terminal membrane-anchoring tail (TM) to eliminate interference by this hydrophobic anchor with the channel forming activity of other portions of the polypeptide during *in vitro* studies with lipid bilayers. All known mammalian BCL-2 family members other than BID and BAD contain a TM (Fig. 10). A polypeptide lacking the TM (ΔTM) can be prepared by expression from a cDNA in which the DNA sequences encoding the TM have been removed from a cDNA encoding the full-length BCL-2 family member using standard deletion mutagenesis techniques. cDNA clones encoding full-length BCL-2 family member proteins are either publicly available or readily obtainable using published sequences.

A preferred anti-apoptotic polypeptide for forming the channel is Bcl-2ΔC21 which comprises amino acids 1-218 or 1-221 of human or mouse Bcl-2 shown in FIG. 11 or a conservatively substituted variant thereof. For pro-apoptotic channels, the preferred pro-apoptotic polypeptide is BaxΔC19, which comprises amino acids 1-173 of human or mouse BAX as shown in FIG. 11, or a conservatively substituted variant thereof.

The channel can be formed by adding the polypeptide to the aqueous solution on either or both sides of the lipid membrane and then mixing the solution. Preferably, the

polypeptide is added to only one side of the lipid membrane which is designated as the cis side. Typically, the concentration of the polypeptide in the aqueous solution is between about 5 and 500 nM, preferably between about 10 and 250 nM, more preferably between about 20 and 125 nM, and even more preferably between about 40 and 60 nM. The aqueous solution is buffered to a pH of between about 4.0 and 7.2. Because low pH promotes insertion into planar lipid bilayers of polypeptides from the BCL-2 family, the aqueous solution is preferably between pH 4.0 and 6.0. The pH of the solution is preferably adjusted to between 6.8 and 7.2 before determining the ion selectivity of the channel.

The polypeptide is preferably incorporated into the planar lipid bilayer at neutral pH by fusing the bilayer with a reconstituted unilamellar proteoliposome comprising the polypeptide. Proteoliposomes can be prepared from purified polypeptides and lipids by standard techniques known in the art, including dialysis, gel filtration and dilution techniques. A membrane fragment vesicle preparation of an intracellular membrane where the BCL-2 family member polypeptide is found *in vivo* can also be used as a source of proteoliposomes.

Preferably, the dialysis approach is used and involves adding about 7.5 to 15 μmole of a polypeptide from the BCL-2 family to about 25 to 75 mg lipid in a buffered solution at a pH of about 6.8 to 7.2. This protein-lipid solution is placed in a dialysis bag with a molecular weight cutoff size selected to make the dialysis bag impermeable to lipid and polypeptide but is permeable to any detergent in the polypeptide preparation. Dialysis is performed in a large volume of aqueous solution buffered at about pH 6.8 to 7.2 until unilamellar proteoliposomes form, usually about 10 to 20 hours.

Once prepared, the proteoliposomes are added to one or both sides, preferably the cis side, of a pre-established planar bilayer with mixing until fusion occurs. As an alternative or in addition to mixing, fusion can be initiated by electrofusion, in which a high electric field pulse is applied to the bilayer chamber. Preferably, about 5-20 μl of proteoliposomes, comprising about 2.0×10^{-4} to 10×10^{-4} nmoles polypeptide incorporated in Azolectin vesicles, are added to the cis side of a planar lipid bilayer.

Formation of a channel by the polypeptide is verified by applying a voltage and assaying for a current above noise and leak values. Once an initial channel current is identified, soluble polypeptide or proteoliposomes unincorporated in the bilayer can then be removed from the chamber by exchanging the chamber contents with fresh buffer.

Prior to or following addition of the channel-forming polypeptide, the concentration of ions in one or both compartments of the bilayer chamber is adjusted to establish an ion concentration gradient between the compartments so that ion selectivity of the channel can be determined. A concentration gradient means that the concentration of cations

and the concentration of ions on one side of the bilayer are both higher than on the other side of the bilayer. The concentrations of cations and anions on a particular side of the bilayer need not be equivalent to each other but it is preferable that they are approximately equal. If the concentration gradient is set up before addition of the polypeptide, the ion selectivity of the initial currents can be identified.

The ion concentration gradient is generally between 2- and 100-fold cis:trans and preferably is between about 3-fold and 10-fold cis:trans. Alternatively, similar gradients can be set up trans:cis. Examples of ion concentration gradients typically used are 150:15 μM, 450:150 μM, and 1.0:0.5 M.

To determine if a compound of interest has cell death promoting or cell death antagonizing activity, the compound is added to either side of a planar bilayer comprising a channel comprised of an anti-apoptotic or pro-apoptotic polypeptide of the BCL-2 family. The ion selectivity of the channel is determined in the presence of an ion concentration gradient which can be established before or after addition of the compound. If the ion selectivity of an anti-apoptotic channel changes from cation-selective to anion-selective in the presence of the compound, the compound is a cell death agonist. Conversely, if the compound changes the ion selectivity of a pro-apoptotic channel from anion-selective to cation-selective, the compound is a cell death antagonist. Preferably, the compound is tested at several different concentrations to determine the lowest amount that is effective in changing ion selectivity.

The compound's activity can be further characterized by determining whether it affects the conductance pattern and rectification of the channel. These channel properties are assessed by measuring the current at different negative and positive voltages when equivalent ion concentrations are present on both sides of the planar bilayer. Based on the distinct characteristics of Bcl-2 and Bax channels and the behavior of the Bcl-2 channel in the absence and presence of an 11-mer peptide with death agonist activity, it is believed that a compound with death agonist activity will impart a pro-apoptotic conductance pattern and rectification to an anti-apoptotic channel.

In a preferred embodiment, the compound is also tested for its ability to insert into membranes to form a channel and for the effect of pH on any such channel forming activity. This can be accomplished by the incubating the compound with lipid vesicles in solutions at different pH values between 3.5 and 7.0 as described below. If the compound has both cation selectivity and its channel forming activity is affected by pH in a manner similar to that of Bax, the compound is more likely to be pro-apoptotic under conditions that activate native apoptosis agonists. Conversely, if the compound has both anion selectivity and its

channel forming activity at acidic and neutral pH is similar to that of Bcl-2, the compound is more likely to be anti-apoptotic under conditions that activate native apoptosis antagonists.

In one embodiment of the invention, the lipid bilayer comprises a unilamellar proteoliposome loaded with a mixture of cations and anions in known amounts and the effect of a compound on the relative rates of cation and anion efflux from the proteoliposome is determined. The unilamellar proteoliposome can be prepared as described above or by any other technique known in the art. Preferably the mixture of cations and ions comprises equal amounts of K^+ and Cl^-. Cation or anion efflux can be measured with cation- or anion-specific electrodes or alternatively by a dye which reacts with the cation or anion in a concentration dependent manner.

The relative rates of cation and anion efflux rates from the vesicles is compared in the absence and presence of a compound of interest. If the compound induces a faster rate of anion efflux from a proteoliposome comprising an anti-apoptotic channel, it has death agonist activity. Conversely, if the compound induces a faster rate of cation efflux from a proteoliposome comprising a pro-apoptotic channel, it has death antagonist activity.

The invention also provides a method for selecting or identifying apoptosis modulating compounds based on their ability to both form a cation- or anion-selective channel in a lipid bilayer and by the effect of pH on their channel forming activity in lipid vesicles. The lipid bilayer lacks preformed channels and can be a planar bilayer or a liposome loaded with cations and anions. A compound of interest is added to the bilayer and ion selectivity is determined as described in the planar bilayer and proteoliposome experiments described above. Preferably, the method comprises assaying for formation of K^+ and/or Cl^--selective channels.

The effect of pH on channel forming activity is quantified by incubating the test compound with lipid vesicles in separate solutions at pH values between 3.5 and 7.0 (*e.g.*, pH 4.0, 5.0, 6.0, 7.0 and others as required). The lipid vesicles comprise a lipid bilayer surrounding an aqueous interior which contains a channel indicator. The channel indicator is any substance that is detectable upon release from the vesicle. For example, the indicator can be a detectable ion or a substrate that forms a detectable reaction product upon contact with an enzyme located outside the vesicle, or a fluorescent compound such as carboxyfluorescein or fluorescein-dextran that increases intensity when its release from the vesicle removes concentration dependent quenching.

The test compound is incubated with vesicles at the desired pH values for the same length of time. At the end of the incubation period, the amount of channel indicator released at each pH is then determined. The channel indicator is detected by an electrode or

spectrophotometer appropriate for the type of indicator used. If no channel indicator is released, then the compound does not form channels. If the ratio of release at pH 3.5 to that at pH 4.0 ($R_{3.5/4.0}$) is less than 1 and the ratio of release at pH 4.0 to that at pH 5.0 ($R_{4.0/5.0}$) is between 1 and 2, the compound is inserting under pH conditions that favor channel formation by anti-apoptotic compounds. An $R_{3.5/4.0}$ of approximately 1 accompanied by an ($R_{4.0/5.0}$) of greater than 7 indicates the compound is inserting under pH conditions that favor channel formation by anti-apoptotic compounds. This information allows the prediction of channel forming characteristics of compounds having ion selectivities appropriate to pro- or anti-apoptotic action. With compounds that vary in structure it may be useful to assess the pH dependence of channel formation using a broader pH range, for example between pH 3.5 and 8.0.

In other embodiments of the present invention, apoptosis-modulating compounds are selected (and identified) by testing for their ability to either (a) induce the formation of a large pore -- *i.e.*, a pore of about 20 - 35 Å that is capable of transporting cytochrome c and/or FD10 out of mitochondria (when the compound is utilized to induce apoptosis), or (b) inhibit such pore formation (when the compound is utilized to inhibit apoptosis). Testing for this ability is preferably performed by applying the compound to a BCL-2 family pore formed in either a planar bilayer or a proteoliposome as discussed above, then determining whether the pore has the cytochrome c or FD10-transporting characteristics desired. For example, if a compound is being selected or identified for its ability to induce apoptosis, a pore is first formed using a pro-apoptotic BCL-2 family member such as Bax. The test compound is then added to the pore and the pore is tested for its ability to allow cytochrome c or FD10 to pass through. That ability is evaluated, for example, by preparing the vesicles or planar lipid bilayers with labeled cytochrome c, labeled FD10, or a labeled compound of the approximate size of cytochrome c, such as dextran, then determining whether the label can pass through the pore. A preferred label for this method is a fluorescein compound such as fluorescein isothiocyanate.

An alternative method would be to cause the formation of pores in the presence of a compound to be tested as described above and to measure the pore size by any of several known methods for the measurement of pore size. If pores having a size of approximately 20 - 22 Å, preferably about 20 - 29 Å, more preferably about 20 - 35 Å in size are increased in number, the test compound is one that promotes apoptosis. Whereas, if the number of large pores is decreased in the presence of the compound, the compound can be determined to be one that inhibits apoptosis. When it is said that the number of pores of a certain size are

increased or decreased in number in the presence of the compound to be tested, what is meant is that the number of such pores that are formed in the presence of the test compound, as compared with the number of such pores formed under the same test conditions, but in the absence of the test compound.

Other embodiments of this method would be apparent to those skilled in the art in light of the disclosure herein, including the determination of the molecularity of the BCL-2 family members that comprise the pore, as shown in Example 11.

The methods and compositions of the present invention are useful for industrial application because, among other things, they provide remedies for disorders which are mediated by excessive apoptosis or excessive inhibition of apoptosis, including Epstein-Barr virus infection, African swine fever virus infection, adenovirus infection, lymphoproliferative conditions, cancer, arthritis, Crohn's disease, inflammation, autoimmune diseases, immunodeficiency diseases, senescence, neurodegenerative diseases, ischemic and reperfusion cell death, infertility, and poor wound healing. These remedies represent potentially improved treatments for these disorders. Also provided are improved methods for evaluating compounds for their effect on apoptosis.

Preferred embodiments of the invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

EXAMPLE 1

This example illustrates that Bax and Bcl-2 induce the release of ions from synthetic lipid vesicles.

The Bax and Bcl-2 polypeptides used in the ion channel studies reported herein were recombinant murine Bcl-2 and Bax polypeptides lacking the C-terminal signal-anchor membrane targeting sequence (Bcl-2ΔC21, amino acids 1-218 of SEQ ID NO:9 and BaxΔC19, amino acids 1-173 of SEQ ID NO:6). These deletion derivatives, also referred to herein as BaxΔTM and Bcl-2ΔTM, were used to avoid any interference of the C-terminal signal-anchor membrane targeting sequence with the capacity of internal α helices to mediate insertion into artificial lipid bilayers.

To produce Bcl-2ΔC21 and BaxΔC19A, cDNAs encoding amino acids 1-218 of murine Bcl-2 (SEQ ID NO:9) and amino acids 1-173 of murine Bax (SEQ ID NO:6) were cloned into pGEX-KG. The resulting recombinant vectors were transformed into XL-1 cells

and expression of GST-Bcl-2ΔC21 and GST-BaxΔC19A fusion proteins was induced by adding 0.1 mM IPTG. The bacterial pellets were resuspended in lysis buffer (0.5mM EDTA, 1mM DTT, 1% Triton X-100, 0.1mg/ml PMSF, 2 ug/ml aprotinin, 2 ug/ml leupeptine and 1 ug/ml pepstatin A in PBS) and sonicated. After centrifugation at 20,000 x g for 20 minutes, the supernatant was applied to glutathione-agarose beads (Sigma). The beads were washed with buffer and treated with 10 units of thrombin/original liter. Cleaved BaxΔC19 and Bcl-2ΔC21 were eluted from beads and the cleavage reaction was terminated by adding 80 ug of Na-p-tosyl-L-lysine choloromethyl ketone (TLCK). The cleaved eluent was dialyzed against buffer (20 mM Tris pH 8.5, 5mM EDTA, 1mM DTT, 0.1% Triton X-100). To remove the GST protein and incompletely cleaved fusion proteins, the dialyzed preparation was further purified on a monoQ column and the proteins were eluted with a NaCl gradient. The examples described below utilized 3 independent protein preparations of Bax and Bcl-2.

EXAMPLE 2

This example illustrates that Bax and Bcl-2 form channels in planar lipid bilayers at low pH.

Unilamellar vesicles composed of 40% 1,2 dioleoyl phosphatidyl glycerol and 60% 1,2 dioleoyl phosphatidyl choline (Avanti Polar Lipids) were prepared in 100 mM KCl, 2 mM $CaNO_3$ and 10 mM dimethylglutarate, pH 5.0 as previously described (Peterson et al., *J. Membr. Biol. 99*:197-204, 1987). The resulting liposomes were diluted 200-fold to a concentration of 0.05 mg/ml in 100 mM KNO_3, 2 mM $CaNO_3$ and 10 mM dimethylglutarate which was titrated to pH 3.5, 4.0, 4.5, 5.0, 5.5 or 6.0 with NaOH or with acetic acid. BaxΔC19 and Bcl-2ΔC21 were added at a concentration of 500 ng/ml and Cl^- efflux was measured with a Cl^- combination ion selective electrode (Accumet). Triton X-100 (0.1%) was added to release the total encapsulated Cl^- at the times indicated by the arrows in Figure 1. The total amount of Cl^- released was quantitated by a calibration curve produced by successive additions of 25 μM KCl.

As shown in FIG. 1A-D, Bax and Bcl-2 mediated release of Cl^- from KCl loaded lipid vesicles is dependent on pH (Fig. 1B-E). The maximum release of Cl^- by Bax occurs at pH 4.0-4.5, decreasing to 50% at pH 3.5 or 5.0 and to less than 10% at a pH of 5.5 (Fig. 1A). When Bax added to vesicles at pH 6.0 was subsequently shifted to pH 4.0 a rapid release of Cl^- resulted indicating the reversibility of the pH influence (Fig. 1C). Bcl-2 displays a more narrow pH dependence of Cl^- release with complete inactivation occurring by pH 5.0 (Fig. 1B). Shifting from pH 5.0 to 4.0 activated the release of Cl^- by Bcl-2 (Fig. 1D). Thus, both purified Bax and Bcl-2 proteins are capable of pH dependent macroscopic ion release requiring activity of the bulk population of Bcl-2 and Bax proteins.

EXAMPLE 3

This example illustrates the formation of Bax channels in planar lipid bilayers at low pH.

Planar lipid bilayers were prepared from soybean lipids by chloroform extraction of Azolectin Type II (10-20% choline, 80-90% negatively charged lipids) (Sigma). The chloroform was removed with a stream of nitrogen and the lipids stored under N_2 until dissolved in decane at 30 mg/ml. This preparation was then stored under nitrogen. The 0.25 mm orifice of a polystyrene cuvette (Warner Instruments) was pretreated with 2μl of the decane lipid solution and the solvent allowed to evaporate. The cuvette was then placed into a bilayer chamber and connected to Bilayer Clamp BC525-a (Warner Instruments) by Ag/AgCl electrodes via agar salt bridges. Data were collected using an Axoscope (Axon Instruments Software), archived on video tape using a Neurocorder DR-484 (Neuro Data Instruments), and analyzed using Origin (Microcal) and pClamp6 (Axon Instruments Software). Slope conductance was calculated by the method of least squares and the variance is given. Ion selectivities were calculated using the reversal potential and the Goldman equation. The reversal potential means the zero-current potential of a bilayer membrane. The reversal potential is zero with no ion gradient and the magnitude of the potential in the presence of an ion gradient reflects the cation versus anion selectivity of the bilayer.

Bilayers were formed by spreading with a polished glass rod and allowed to thin to a capacitance of 0.4 $\mu F/cm^2$ at which point the noise was typically 0.2 pA and the leak conductance was 20 pS. The salt concentrations were initially 450 mM KCl in the cis chamber (1.0 ml) and 150 mM KCl in the trans chamber (0.5 ml) to permit identification of spontaneous initial currents. Outward (positive) currents were defined as K^+ moving cis-to trans. All solutions were buffered to pH 4.0 with 10 mM K-acetate. BaxΔTM or Bcl-2ΔTM (approx. 1 μg) was added to the cis chamber with mixing. After initial currents were identified the soluble protein was usually removed by exchanging the cis chamber contents with aliquots of buffer.

Four minutes after BaxΔTM was added to the cis chamber of an established planar lipid bilayer in a 450/150 mM (cis to trans) KCl gradient at pH 4.0, an initial, inward current appeared spontaneously (O_S), reflecting Cl^- moving down the KCl gradient (Fig. 2A). The initial current of the Bax channel had consistent characteristics in multiple experiments: it was always inward, usually appeared within 10 minutes following BAXΔTM addition, and had a conductance of 22 (s.d. 5, n = 5) pS.

As shown in FIG. 2B, a characteristic pattern of Bax currents occurred during a transition from O_S to a large open pore. At +40 mV four current levels were noted during a

typical Bax channel transition which took ~5 mins.: 0 pA (C and O_S), 2.36 pA (O_1), 6.4 pA (O_2) and 8.81 pA (O_{1+2}) (Fig. 2B, upper panel). The largest current appears to be the sum of the two smaller levels (O_1 and O_2). The closed (C) and O_S levels are indistinguishable at +40 mV as it is close to the chloride reversal potential (E_R=29mV). Direct transitions between these levels, which occur in both directions, suggest a random mechanism for movement between the open states (FIG. 2B). At –40 mV there were also four levels: 0 pA (C), -2.14 pA (O_S), -10.9 pA (O_1), -30.5 pA (O_{1+2}), but no observable O_S to O_2 transition (FIG. 2B, lower panel). Hyperpolarization to –40 mV increased the channel activity, demonstrating voltage dependent behavior of the channel. Computing the E_R for each of these current levels gives a substantial Cl^- selectivity that averages $P_K/P_{Cl} \cong 0.10$. The final and apparently stable BAX channel at pH 4.0 was Cl^- selective, with a slope conductance of 0.731 ± 0.01 nS and was characteristically open.

This progression of Bax channels proceeded after the removal of protein from the cis chamber. Increasing the pH to 7.0 altered the conductance of the Bax channel to 0.329 ± 0.002 nS in a 450/150 mM KCl gradient or to 0.302 ± 0.008 nS when both chambers were 150 mM KCl (FIG. 2C, 2D). The Bax channel was observed in this activity state at pH 7.0 for prolonged periods. At pH 7.0, the current of the Bax channel was linear with voltage and retained a mild selectivity for Cl^- (P_K/P_{Cl}=0.5).

EXAMPLE 4

This example illustrates the formation of Bax channels in planar lipid bilayers at pH 7.0 following fusion of the planar bilayer with unilamellar proteoliposomes reconstituted with BaxΔTM.

Proteoliposomes for fusion with a planar lipid bilayer were prepared as follows. Purified protein (0.25 μg) was added to 50 mg lipid (azolectin) in 20 μl of KCl buffer (150 mM KCl, 10 mM HEPES, pH 7.0). This mixture was placed into a dialysis bag with a molecular weight cutoff of 12 KDa. After 16 hours dialysis in 3000 volumes of 150 mM KCl (10 mM HEPES, pH 7.0) the proteoliposome suspension was removed and placed on ice for immediate use. The bilayer was set up with a 450:150 cis to trans KCl gradient to identify the ion selectivity of the initial currents. Fusion was initiated by adding 5 – 20 μl of lipid vesicles (only 5 – 20 ng of protein) to the cis bilayer chamber with mixing. Non-fused proteoliposomes were removed from the compartments and solutions exchanged as described above.

BaxΔTM was incorporated into lipid vesicles using standard techniques. The resulting Bax proteoliposomes were added to the cis chamber of an established lipid bilayer with a 450/150 mM KCl gradient at pH 7.0 to permit identification of initial currents. The

inward ($P_K<P_{Cl}$) current observed was characteristically open and large. Transitions between one and two channels open ($0 \rightarrow 0^2$, Fig. 3A) that were identical in amplitude suggested that 0 represented the single channel conductance (Fig. 3A). A mild Cl^- selectivity ($P_K/P_{Cl} = 0.32$) was calculated from the reversal potential.

The concentration of KCl was adjusted to 150 mM KCl in both cis and trans compartments and voltage dependence of the current was determined when one channel was present (Fig. 3B and 3C). Mild outward rectification in the symmetrical KCl solution was observed. At negative voltages the channel conductance averaged 1.5 (sd 0.3, n=3) nS (FIG. 3C), while at positive voltages (+70 mV) the rapid flickering was consistent with channel block as a mechanism for the rectification seen in FIG. 3B. At –70 mV there was no flickering but periodic closing of 20-30 msec duration was observed (Fig. 3C), a pattern similar to that reported for porin type channels (Benz, *Biochim. Biophys. Acta 1197*:167-196, 1994).

EXAMPLE 5

This example illustrates the formation of Bcl-2 channels in planar lipid bilayers at low pH.

Bcl-2ΔTM was added to the cis chamber of an established bilayer in a 450/150 mM KCl gradient at pH 4.0. An initial outward ($P_K/P_{Cl} = 3.9$) current (O_S) consistently appeared within 5 minutes in multiple experiments (Fig. 4A). The magnitude of the current was 0.85±0.06 pA which flickered open and shut in the first few seconds and subsequently remained open. Under these conditions, the Bcl-2 channel had a conductance of 80.3 ± 0.06 pS (Fig. 4D).

Subsequently, the Bcl-2 channel remained open for long periods (5-10 seconds) with brief closures (Fig. 4B, 4C). This initial state was present for only 2 – 5 minutes under these conditions and then the Bcl-2 channel progressed to a stable open pore at pH 4.0 with a 1.90 ± 0.06 nS conductance (Fig. 4E). After the pH was shifted to 7.0, the Bcl-2 channel remained a large pore with a 2.14 ± 0.04 nS conductance and K^+ selectivity of $P_K/P_{Cl} = 6.5$ (Fig. 4E).

EXAMPLE 6

This example illustrates the formation of Bcl-2 channels in planar lipid bilayers at pH 7.0 following fusion of the planar bilayer with unilamellar proteoliposomes reconstituted with Bcl-2ΔTM.

Bcl-2ΔTM proteoliposomes were prepared as described in Example 4 and added to the cis chamber of an established bilayer with a 450/150 mM KCl gradient. In multiple experiments fusion of Bcl-2 proteoliposomes to the bilayer always resulted in an outward (K^+) current (Fig. 5A). The closures in Fig. 5A indicate a single channel current of 5 pA (O),

although multiple channels could open simultaneously (O^2). When a series of voltage steps were applied to a single channel established in the bilayer, channel closures were observed at voltages greater than ± 50 mV (Fig. 5B). These closures became more complete as the voltage increased.

The voltage dependence of the open channel current in symmetrical 150 mM KCl was linear with a slope conductance of 1.08 (s.d. 0.10, n = 5) nS (FIGS. 5C and 5D). However, with hyperpolarization to more than –70 mV, a channel closure occurs (Fig. 5C). The reversal potential in KCl gradients indicated a mild K^+ selectivity (P_K/P_{Cl}=2.4). The small channels and time dependent changes noted when Bcl-2ΔTM was inserted into planar lipid bilayers at low pH were not observed when reconstituted proteoliposomes containing Bcl-2ΔTM were fused to planar lipid bilayers at pH 7.0.

EXAMPLE 7

This example illustrates models of the three-dimensional structure of the α-5 and α-6 helices of Bax and Bcl-2.

The models were generated using INSIGHTII (Biosym, San Diego) from the crystallographic model of Bcl-x_L (PDB entry 1MAZ). Views of the positively and negatively charged surfaces of the α-5 and α-6 helices of Bax and Bcl-2, respectively, were calculated and displayed as shown in FIG. 6 using GRASP (Nicholls et al., *Protein Struct. Funct. Genet. 11*:281-296, 1991). The darker surfaces represent the most highly charged regions.

The experiments described in Examples 1-6 demonstrate that Bax and Bcl-2 have distinct channel forming properties. The inventors herein found that both Bax and Bcl-2 initiate rapid release of ions from liposomes when added at low pH. However, Bax demonstrated a broader pH optimum, retaining activity as high as pH 5.5. It is believed this could be due to the higher α5-helix pI of 10.64 for Bax versus 4.55 for Bcl-2 (FIG. 6). If the insertion of the putative transmembrane α5, α6-helices of these apoptotic regulators benefits from charge reduction, the lower pH requirement for Bcl-2 may reflect glutamic acid residues that would be prone to ionization with increasing pH in contrast to the presence of lysine and arginine residues in Bax (FIG. 6).

The ion channels formed by Bax and Bcl-2 in planar lipid bilayers have characteristics that depend in part on the method of incorporation. When BaxΔTM or Bcl-2ΔTM is inserted into bilayers at low pH the initial currents were small with conductances of 22 pS and 80 pS respectively. Like Bcl-x_L (Minn et al., *supra*), and consistent with other observations on Bcl-2 (Schendel et al, *supra*), the experiments described herein showed a mild cation selectivity for anti-apoptotic Bcl-2. However, the pro-apoptotic molecule Bax surprisingly has a

consistent anion selectivity. If the α5, α6-helices contribute to the channel these selectivities may reflect the positively charged residues of Bax and negatively charged residues of Bcl-2. While modest differences in ion selectivity are unlikely to be the sole explanation for opposite influences on apoptosis, these charge reversals appear to be consistent in the α5, α6-helices of anti- versus pro-apoptotic members (Fig. 7). Bax channels respond to shifting the pH to 7.0 after insertion at pH 4.0 consistent with previous observations on toxins and porins (Mindell et al., *Biophys. J. 62*:41-44, 1992 and Todt et al., Biochem. 31, 10471-10478, 1992). The changes described herein could also relate to pH dependent ionization of charged residues in these channels.

A striking progression of the Bax channel in planar bilayers occurred within 2-4 minutes of its initial appearance. This included i) an early Cl^- selective small channel, ii) a transition phase with multiple subconductance levels and moderate Cl^- selectivity, and iii) an apparently stable ohmic pore of large conductance that is mildly Cl^- selective and open continuously (Fig. 2). The Bcl-2 channel activity also progressed from an early K^+ selective small channel that opened and closed spontaneously to a large ohmic pore (Fig. 4). Removal of protein from the chamber and alteration of salt concentrations did not prevent Bax and Bcl-2 channel transition which may represent intra-membranous organization of Bcl-2 or Bax into its mature form. Of note, shifting from pH 4.0 to 7.0 altered the conductance and selectivity of Bax, but not Bcl-2. In contrast, the overnight reconstitution of Bax or Bcl-2 into lipid vesicles which were subsequently fused to planar bilayers resulted immediately in large, open pores (Fig. 3B and 5C). In addition to differences in ion selectivity, Bcl-2 and Bax channels display other unique characteristics, including conductance, voltage dependence and rectification.

EXAMPLE 8

This example illustrates that the conductance pattern of a Bcl-2 channel is altered by a Bax peptide death agonist (SEQ ID NO:1) to appear similar to that of a Bax channel.

Control bilayers containing Bcl-2 or Bax channels were prepared by fusion of unilamellar proteoliposomes with incorporated Bcl-2ΔTM or BaxΔTM with an established planar bilayer as described in Example 4. Test bilayers containing Bcl-2 channels were prepared in the same manner to which an 11 mer Bax peptide (SEQ ID NO:1) with known death agonist activity was added at 20, 50 or 100 μM. A voltage of 80 mV was applied to each control and test channel and its conductance measured for a period of 12 to 16 seconds and the frequency that each channel was measured at a particular conductance level (pA) was calculated. The data, representing about 500,000 measurements, are shown as histograms in FIGS. 8A-8E.

As seen in FIG. 8A, the Bcl-2 control channel moves between several conductance levels, clustering around preferred open states at about 80 pA, 102 pA, and 130 pA. Bax displays a simpler conductance pattern, with two preferred conductance levels between about 45 and 55 pA (FIG. 8E).

The addition of the 11 mer Bax peptide to a Bcl-2 channel changes its characteristic conductance pattern in a progressive manner as the concentration is increased from 20 to 100 μM (FIG. 8B-D). At 100 μM the single remaining peak is not coincident with any of the levels seen in Bcl-2 but coincides with one of the less frequent Bax peaks at about 65 pA. When murine T cell hybridoma 2B4 cells are treated with 100 μM of this peptide fused with the HIV tat peptide, which increases cell uptake of the Bax peptide, a significant number of the cells are dead in less than 4 hr as compared to treatment with the tat peptide alone (data not shown). Thus, the complex conductance pattern of a Bcl-2 channel is altered to a simpler Bax-like conductance pattern by the same concentration of peptide that promotes cell death *in vitro*.

The activity of Bax channels in the lipid bilayer was not affected by addition of the Bax 11-mer peptide at concentrations between 20 and 100 μM. In addition, the Bax 11-mer peptide exhibited no channel forming activity upon addition to planar lipid bilayers which lack Bcl-2 related proteins.

EXAMPLE 9

This example illustrates the effect of the Bax death agonist peptide (SEQ ID NO:1) on ion selectivity and conductance of a Bcl-2 channel, as well as the effect of the Bcl-2 death antagonist (SEQ ID NO:2) and full length Bcl-2 on ion selectivity of a Bax channel.

Bilayers containing Bcl-2 and Bax channels were prepared as described in Example 4 with 150 mM KCl on both sides of the bilayer. The Bax death agonist peptide (SEQ ID NO:1) was added to the cis side of a Bcl-2 channel at 150 μM. The voltage dependence of the currents and the ion selectivity for the Bcl-2 channel in the absence and presence of the death agonist peptide, as well as the Bax channel were determined and the data are shown in FIGS 9A-C.

The IV plots show that the death agonist peptide imparted rectification to the voltage dependence of the Bcl-2 channel current, similar to the rectification displayed by the Bax channel, as indicated by the curving lines at positive potentials in the plots in FIGS. 9B and 9C. The slope of Bcl-2 channels in the presence of the death agonist peptide and at negative potentials is linear and intermediate between that of Bcl-2 and Bax alone. Of note, addition of the death agonist peptide reversed the ion selectivity of the Bcl-2 channel from K^+ selective

with a pK/pCl=2.4) to Cl- selective with a pK/pCl value, 0.26±0.05, within the range of that measured for the Bax channel (0.32 pK/pCl).

The insertion of Bax into a planar lipid bilayer produced a Cl^- selective channel with 1.5 nS conductance and P_K/P_{Cl} of 0.3. When full length Bcl-2 (SEQ ID NO:9) was added to the bilayer, the conductance increased to 2.5 ± 0.5 nS and the P_K/P_{Cl} shifted to 1.4 ± 0.5.

When 50-200 μM Bcl-2 death antagonist 11-mer (SEQ ID NO:2) was added to the Bax-containing planar lipid bilayer, currents at positive potentials decreased consistent with a shift towards cation selectivity.

EXAMPLE 10

This example illustrates that the ion conductance patterns of planar lipid bilayers containing Bad or Bid pro-apoptotic BCL-2 family members are similar to Bax-containing bilayers, and that a loss-of-function Bax variant which does not induce apoptosis has ion conductance characteristics similar to anti-apoptotic BCL-2 family members.

Bad channels in azolectin, pH 7.0, were formed and conductance characteristics of these channels were characterized as disclosed in Example 4. The $Cl^-:K^+$ selectivity of this composition was 4:1, which is very similar to the selectivity established for Bax channels. The conductance of the channels were about 1 nS with gating similar to Bax channels.

Bid channels were also formed as with the Bad channels discussed above. A $Cl^-:K^+$ selectivity of 3.7:1, with a maximum conductance of 4 nS was established. The channel closed in steps of 1 nS. These characteristics are also similar to those of Bax channels.

A loss-of-function form of Bax ($ScBax^{G67R}$) has been isolated from human lymphoma cells (Meijerink et al., *Leukemia 9*:1828-1832, 1995; Meijerink et al., *Blood 91*:2991-2997, 1998). This Bax is unable to promote apoptosis due to an amino acid change from gly to arg at position 67 (within the BH3 region). When incorporated into planar lipid bilayers as in Example 4, $ScBax^{G67R}$ formed channels having the gating and size of wild-type Bax, but with $K^+:Cl^-$ selectivity of 3.2:1, which is distinct from channels formed with wild-type (pro-apoptotic) Bax, and similar to channels formed with anti-apoptotic BCL-2 family members.

EXAMPLE 11

This example illustrates the characterization of the kinetics and size of the apoptotic pore formed by Bax, and, in particular, establishes that Bax can act alone to form a pore that can act as a channel capable of conducting cytochrome c.

Selected death signals activate pro-apoptotic BAX, resulting in translocation to mitochondria where it inserts as an integral membrane, oligomeric protein. See, *e.g.*, Gross, A., McDonnell, J. M. & Korsmeyer, S. J. *Genes Dev* **13**, 1899-1911 (1999); Wolter, K. G. *et al. J Cell Biol* **139**, 1281-1292 (1997); and Gross, A., Jockel, J., Wei, M. C. & Korsmeyer, S.

J. *Embo J* **17**, 3878-3885 (1998). Mitochondrial dysfunction follows with the release of cytochrome c and the activation of caspases. See, *e.g.*, Zou, H., Li, Y., Liu, X. & Wang, X. *J Biol Chem* **274**, 11549-15556 (1999); Finucane, D. M., Bossy-Wetzel, E., Waterhouse, N. J., Cotter, T. G. & Green, D. R. *J Biol Chem* **274**, 2225-2233 (1999); Pastorino, J. G., Chen, S. T., Tafani, M., Snyder, J. W. & Farber, J. L. *J Biol Chem* **273**, 7770-7775 (1998); and Xiang, J., Chao, D. T. & Korsmeyer, S. J. *Proc Natl Acad Sci U S A* **93**, 14559-14563 (1996). In planar lipid bilayers BAX forms ion conducting channels and will release chloride or carboxyfluorescein from artificial liposomes. See, *e.g.*, Schlesinger, P. H. *et al. Proc Natl Acad Sci U S A* **94**, 11357-11362 (1997); and Antonsson, B. *et al. Science* **277**, 370-372 (1997). The ion transmitting pore formed by BAX can progress to a conductance of ~1.5 nS with low ion selectivity. Models propose that BAX interacts with other pore forming molecules or that BAX generally disrupts the outer mitochondrial membrane. See, *e.g.*, Basanez, G. *et al. Proc Natl Acad Sci U S A* **96**, 5492-5497 (1999); and Shimizu, S., Narita, M. & Tsujimoto, Y. *Nature* **399**, 483-487 (1999). In this example we show nanomolar BAX rapidly forming membrane pores (See, *e.g.*, Miller, C. *Annu Rev Physiol* **46**, 549-558 (1984); and Chen, R. F. & Knutson, J. R. *Anal Biochem* **172**, 61-77 (1988)) which released intravesicular carboxyfluorescein, FITC-dextran or FITC-cytochrome c, and that are blocked by dextran molecules of defined size. A concentration—dependent progression from a pore of 10.7±3.2 Å, consisting of two BAX molecules, to a 22.1±3.9 Å pore, requiring four BAX molecules was observed. The larger BAX pore transported cytochrome c.

The BAX release of 6-carboxyfluorescein from 200 nm unilamellar vesicles was analyzed by using recombinant, purified, functionally identical BAXΔC19 molecules that were monodispersed at pH 7.2. See, *e.g.,* Antonsson, B. *et al. Science* **277**, 370-372 (1997); Priault, M., Chaudhuri, B., Clow, A., Camougrand, N. & Manon, S. *Eur J Biochem* **260**, 684-691 (1999); Antonsson, B., Montessuit, S., Lauper, S., Eskes, R. & Martinou, J. C. *Biochem J* **345 Pt 2**, 271-278 (2000); Schwarz, G., Zong, R. T. & Popescu, T. *Biochim Biophys Acta* **1110**, 97-104 (1992); and Rex, S. *Biophys Chem* **58**, 75-85 (1996). Prior studies of the melittin pore indicate that an exponential dequenching reflects the concentration dependent activation of oligomeric pores. See. *e.g.*, Miller, C. *Annu Rev Physiol* **46**, 549-558 (1984); Schwarz, G., Zong, R. T. & Popescu, T. *Biochim Biophys Acta* **1110**, 97-104 (1992); Rex, S. *Biophys Chem* **58**, 75-85 (1996); Schwarz, G. & Robert, C. H. *Biophys Chem* **42**, 291-296 (1992); and Schwarz, G. & Robert, C. H. *Biophys J* **58**, 577 (1990). The exponential kinetics of BAX pore activation and are well described by equation (1), and the fit was not improved by additional exponential terms. Specifically, dequenching F_{520}, was computed by $F_{520} = F - F_0 / F_{Triton} - F_0$, with F_0 and F_{Triton} being the zero time and Triton X-100 produced

flourescence. The time dependence of dequenching was fitted to equation (1) where A_1 is the size of the exponential component; τ is the time constant for the exponential component; m is the slope of the linear component. Data were fit using the Levenberg-Marquardt algorithm which generated χ^2 less than 0.05 and p values from 0.05 to 0.001 depending upon the number of trials.

$$F_{520} = F_0 + A_1\left(1 - e^{-(time/\tau)}\right) + m * time \qquad (1)$$

Fitting the time dependence of BAX pore mediated dequenching (Figure 12) to equation (1) yielded an exponential time constant, τ, that characterized the pore activation kinetics.

Carboxyfluorescein vesicles were prepared and diluted as follows: Liposome composition for all experiments was 30% 1 ,2-Dioleoyl-sn-Glycero-3-phosphate and 70% 1 ,2-Dioleoyl-sn-Glycero-3-phosphocholine (Avanti Polar Lipids, Al). The lipid was stored *in vacuo* at -20°C after evaporation of chloroform under N_2 gas. These were hydrated in 500 µl of 10mM HEPES, 100mM KCl, 1mM EDTA, pH 7.4 containing the fluorescein labeled molecules (carboxyfluorescein at 50 mM [CF], FITC-cytochrome c at 10 mg per ml [FCC], or FITC-dextran at 20 mg/ml [FD10]). Hydrated lipids were treated by 5 freeze thaw cycles and sonicated for 30 seconds x 5 in a 1600 watt bath sonicator. Vesicles were sized to 200 nm using polycarbonate filters (Nucleopore) and separated from unincorporated fluorescein compounds by Sephacryl S-300 (Sigma) chromatography. Liposome size was determined by dynamic light scattering using Coulter Submicron Particle Analyzer, model N4MD, Hialeaha Fl. CF vesicles were used at $2.2x10^{10}$ vesicles per ml and FCC or FD10 vesicles were diluted to $2.2x10^{11}$ vesicle per ml. Molecular sizes were determined by dynamic light scattering using a Dyna Pro-801TC, Protein Solutions, Inc., Charlottesville, VA.

BAXΔC19 was prepared as follows: GST-BAXΔC19 was expressed *in vitro* and cleaved with thrombin. (Schlesinger, P. H. *et al. Proc Natl Acad Sci USA* **94**, 11357-11362 (1997)). The BAXΔC19, 1-3 µg/µml was denatured in 6 M Urea solution containing 0.5% n-octyl-13-β-glucopyranoside, 0.1 mM EDTA, 100mM KNO3, 10mM HEPES, pH7.2 on ice for 30 minutes. Renaturation was by dialysis using buffer without urea at pH 7.2 and 0.5% octyl-13-β-glucopyranoside (below the critical mice concentration) diluted by 10^6 over 24 hours at 4°C yielding a monodispersed protein of 21 Å as determined by dynamic light scattering.

At t=0 the indicated concentration of BAXΔC19 was added with mixing and the fluorescence followed at excitation of 497 nm and emission of 520 nm. Figure 12(A) shows the time dependence of fluorescence dequenching at selected BAX concentrations, • 1.82 nM,

x 9.09 nM, Δ18.2 nM, and ■ 36.4 nM. Each average curve (n=3) was fitted to equation (1) with a $p < 0.05$. The time constants, τ, are listed above the plot. Figure 12(B) shows a Hill plot of the velocity of dequenching with increasing BAX concentrations. The slope of this plot at 0.2Vmax is 2.0±0.19 rising to 3.9±0.24 at $0.8V_{max}$. The inset indicates the continuous slope across the studied BAX concentrations. Error bars indicate the standard deviation. Figure 12(C) shows the inhibition of carboxyfluorescein dequenching at 9 nM BAX by 20 μM dextrans of 6, 9 and 39.4 kDa. The dequenching time constant, $\tau_{blocked}$, reflects the size-specific dextran block of carboxyfluorescein release (± standard deviation). The exponential phase of dequenching is presented after removal of the linear release [equation (1)]. The time axis includes a break so that the final levels of the exponential dequenching can be clearly shown to be identical. Figure 12(D) shows a comparison of functional pore diameter determined by size-dependent dextran inhibition of dequenching at three BAX concentrations; 5 nM (▽), 9 nM (□) and 20 nM (Δ). Time constants are averaged (n=3) and plotted with standard deviation at each dextran size. At each BAX concentration size-dependent dextran inhibition was fitted to a Gaussian peak and the peak center ± 1 standard deviation was determined (5 nM: 10.7±3.2 Å, 9 nM: 22.1±3.1 Å, and 21.4±5.2 Å). The vertical hatched column for each BAX concentration reflects the fitted peak with a width of two standard deviations.

Rapid exponential dequenching, which was strongly dependent on BAX concentrations, occurred at nanomolar levels (Figure 12A) and was sensitive to heat or protease inactivation. BAX dequenching maximized at the unilamellar content of the vesicle preparations as determined by dynamic light scattering and electron microscopy. Furthermore these protein to lipid ratios do not typically form the lipid disks required for the lysis of intravesicular lamellae (Dufourcq, J. *et al. Biochim Biophys Acta* **859**, 33-48 (1986)). This strong concentration dependence indicated that more than one BAX molecule was required for pore activation. Therefore Hill plots of the velocities of dequenching were analyzed to determine the molecularity of pore activation as BAX concentration increased (Figure 12B). See, *e.g.*, Segel, I. H. 371-385 (John Wiley & Sons, 1975). It should be noted that the Hill plot is curved in the range 0.2 to 0.8 of V_{max} with the slope increasing from 2.0±0.19 to an apparent maximum of 3.9±0.24 (see inset to Figure 12B). Thus, the number of BAX molecules participating in the active membrane pore increases in a concentration dependent fashion up to an apparent maximum of 4. The capacity of BCL-2 family members to form dimers has been a prominent characteristic of their activity in cells, and death signals increase the fraction of BAX dimers and higher oligomers recovered from mitochondria. See, *e.g.*, Gross, A., McDonnell, J. M. & Korsmeyer, S. J. *Genes Dev* **13**, 1899-1911 (1999); Wolter, K.

G. *et al. J Cell Biol* **139**, 1281-1292 (1997); and Gross, A., Jockel, J., Wei, M. C. & Korsmeyer, S. J. *Embo J* **17**, 3878-3885 (1998).

Transport kinetics are used to characterize the diffusion pathway in classic channels (Renkin, E. M. *J Gen Physiol* **38**, 225 (1954)). Restricted diffusion in the pore increases transit time as the penetrating molecule approximates the pore diameter. (Levitt, D. G. *Curr. Topics In Membranes and Transport* **21**, 181 (1984)). Accordingly we employed unlabelled dextran molecules as blockers of carboxyfluorescein passage through the BAX pore, using the effectiveness of various sized dextrans as a measure of the pore diameter. See, *e.g.*, Levitt, D. G. *Curr. Topics In Membranes and Transport* **21**, 181 (1984); and Hille, B. *J Gen Physiol* **51**, 199-219 (1968). For 9 nM BAX and 9 kDa dextran the dequenching time constant, $\tau_{blocked}$, increased by a maximum factor of 2.75—fold (Figure 12C). This indicates that the Stokes diameter of 9 kDa dextran, 19±4 Å, is closest to the pore diameter at this BAX concentration. See, *e.g.*, Renkin, E. M. *J Gen Physiol* **38**, 225 (1954); Levitt, D. G. *Curr. Topics In Membranes and Transport* **21**, 181 (1984); and Hille, B. *J Gen Physiol* **51**, 199-219 (1968). In Figure 12C the linear component of dequenching was removed to make clear that 6 and 9 kDa dextran slowed the rate of dequenching but not the extent and that 39.4 kDa dextran, Stokes diameter of 36±9 Å, was too large to inhibit dequenching. Size-dependent inhibition by dextrans was detrermined at 5, 9 and 20 nM BAX and optimal blocking size observed to increase with BAX concentration (Figure 12D). These analyses indicate a dominant pore size of 10.7±3.2 Å at 5 nM BAX where the slope of the Hill plot is 2±0.2 indicating a bimolecular mode of pore activation. The pore size reached a maximum of ~22 Å at 9 nM BAX (Figure 12D) where the slope of the Hill plot is 3.9±0.24 reflecting a shift in the number of participating BAX molecules to ~4. No further increase in pore size was noted at 20 nM BAX consistent with Figure 12 where the molecularity of pore activation plateaued at ~4

We selected a fluorescein-dextran conjugate of defined size, 10 kD FITC-dextran (FD10), with a Stokes diameter of 22±5 Å to establish a dequenching assay for the transmission of macromolecules. Vesicles were prepared using 20 mg/ml FD10 and dequenching initiated by the addition of 72.7 nM renatured BAX. In Figure 13A it is shown that increasing fluorescence resulted upon release of FD10 from the vesicles as predicted by the Stern-Volmer analysis of concentration dependence of FITC-dextran fluorescence described in Methods. Dequenching was reduced by the addition of the indicated concentrations of extravesicular unlabelled 9 kDa dextran. Each time course represents the average of three experiments and was fitted to equation (1) as described. The time axis includes a break so that the final levels of the dequenching can be clearly shown to be identical. In Figure 13B, the time constants for 0,10, 30 and 40 μM blocking dextran are

plotted and fitted to a sigmoid curve and the IC50 for 9 kDa dextran block of FITC-dextran release was determined to be $4.05\pm0.46x10^{-5}$.

Unlabelled 9 kDa dextran slowed but did not reduce total BAX-mediated dequenching demonstrating that pore formation, not lysis, was the mechanism of FD10 release (Figure 13A). The IC_{50} for 9 kDa dextran block was $4.05\pm0.46x10^{-5}$M (Figure 13B), which closely approximates the intravesicular FD10 concentration calculated from the Stern-Volmer analysis, ~50 μM. (See, Badley, R. A. in *Modern Fluorescence Spectroscopy* 112-119 (Plenum Press, New York, 1976); and Eftink, M. R. & Ghiron, C. A. *Anal Biochem* **114**, 199-227 (1981). Therefore we conclude that the large homotypic BAX pore provides a diffusion pathway for the movement of appropriately-sized macromolecules.

Given the capacity of the BAX pore to release FD10, a 22 Å macromolecule, we prepared liposomes containing ~25 μM FITC-labelled cytochrome c (FCC), as determined by Stern-Vollmer analysis. (Badley, R. A. in *Modern Fluorescence Spectroscopy* 112-119 (Plenum Press, New York, 1976)).

FITC cytochrome c was prepared as follows: Holocytochrome c (10 mg) and fluorescein isothiocyanate (5 mg) were dissolved in one ml 100mM KCl, 10mM HEPES, pH 7.2 at room temperature. After 30 minutes insoluble FITC was removed by centrifugation and the supernatant was dialyzed at 4°C changing buffer until the dialyzate was free of fluorescence. FD10 (Sigma, St. Louis, MO) and FCC fluorescence were characterized by Stern-Volmer analysis at emission (520 nm) and excitation (497 nm) maxima to define quenching coefficients, Ksv, of $4.42\pm1.24x10^4$ M^{-1} and $1.95\pm0.34x10^4$ M^{-1} respectively. (See, Eftink, M. R. & Ghiron, C. A. *Anal Biochem* **114**, 199-227 (1981).

Dynamic light scattering estimated a Stokes diameter for FCC of 20±5 Å, while native cytochrome c was 17±3 Å. As for FD10, the FCC assays were done with increased vesicle mass and BAX concentrations, producing greater dequenching rates for FCC than the carboxyfluorescein assays (Figure 14A).

Vesicles were prepared with 10 mg/ml FCC as described above and dequenching was initiated by the addition of BAX. Figure 14A shows a comparison of the time course of dequenching for carboxyfluorescein (1.82 nM renatured BAX) and FCC containing vesicles (322 nM renatured BAX). The time constants, τ, for dequenching were 77.1±0.7 and 5.01±2.7 seconds respectively. In Figure 14B, the effect of extravesicular unlabelled cytochrome c on BAX-dependent FCC release was determined using 322 nM BAX. Time constants for dequenching were determined by fitting to equation 1 and are plotted on a logarithmic scale in the inset panel. This plot was fitted to a sigmoid function yielding an 1C50=9.05±0.2x104. In Figure 14C, the time constant for dequenching of FCC was determined at 72 to 540 nM BAX

and used to construct a Hill plot from 0.95 to 0.05V_{max}. A linear plot with a slope of 4 is presented along with the standard error (n=3) for each experimental point. In Figure 14D, the size—specific dextran block, $\tau_{blocked,}$ of BAX mediated FCC dequenching was determined as described in Figure 13. The peak and standard deviation were estimated by Gaussian fitting to be 28.9±6.0 Å and dextrans with a Stokes diameter greater than this show decreasing inhibition. Statistics and curve fitting. Each averaged experiment (n=3) was fitted to equation (1) and subjected to χ^2 analysis. Extra-vesicular unlabelled cytochrome c proved capable of inhibiting the release of FCC (Figure 14B). Analysis of the FCC concentration dependence of $\tau_{blocked}$ produced an IC_{50} of 9.05±0.2x10^{-6} M (inset of Figure 14B). Of note this is significantly below the intravesicular concentration of FCC which we have determined from its Stern-Volmer constant. The inhibition by cytochrome c contrasts with that of 10 kDa dextran where the IC_{50} approximated the intravesicular FD10 concentration, as is expected for diffusional size-dependent pore transport. The inhibitionby cytochrome c confirms a pore mechanism of release and suggests the possibility of a specific interaction with the BAX pore.

A Hill plot of the velocity of FCC dequenching (Figure 14C) revealed a uniform slope of 4 across the effective concentrations of BAX, indicating that only the large 22 Å BAX pore is responsible for release of cytochrome c,. Next, we calibrated the FCC pore by size-specific dextran inhibition of FCC release at a BAX concentration with a pore activation molecularity of 4. In Figure 14D the time constant, $\tau_{blocked}$ is plotted against the Stokes diameter of the tested dextrans. Analysis by fitting to a Gaussian function indicated a pore size of 29.8±6.0 Å. This is consistent with the large BAX pore observed for carboxyfluorescein release (Figure 12B), although the slightly larger predicted size might also reflect a specific interaction. From these data it can be concluded that BAX at nanomolar concentrations rapidly forms a pathway for cytochrome c release from liposomes that does not require additional proteins.

The biologic importance of BAX multimerization was supported by experiments in which the enforced dimerization of an FKBP-BAX chimeric molecule proved sufficient to kill cells. (Gross, A., Jockel, J., Wei, M. C. & Korsmeyer, S. J. *Embo J* **17**, 3878-3885 (1998)). Other models suggest BAX interacts with resident mitochondrial membrane pores, such as VDAC, or propose the lytic disruption of the outer mitochondrial membrane. See, *e.g.,* Basanez, G. *et al. Proc Natl Acad Sci U S A* **96**, 5492-5497 (1999); and Shimizu, S., Narita, M. & Tsujimoto, Y. *Nature* **399**, 483-487 (1999). However, BAX alone can form a membrane pathway sufficient to transport cytochrome c. The subsequent release of larger proteins, such as sulfite oxidase, suggests that further oligomerization of BAX *in vivo* or secondary effects may follow. (Antonsson, B., Montessuit, S., Lauper, S., Eskes, R. & Martinou, J. C.

Biochem J **345 Pt 2,** 271-278 (2000)). Based on the data here, we believe that the BAX mediated release of cytochrome c from mitochondria requires the establishment of a sufficient density of BAX molecules in the mitochondrial membrane to form the large pore. In this way BAX pore activation plays a critical role in the commitment to cell death.

In view of the above, it will be seen that the several advantages of the invention are achieved and other advantageous results attained.

As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

All references cited in this specification either *supra* or *infra* are hereby incorporated by reference. The discussion of the references herein is intended merely to summarize the assertions made by their authors and no admission is made as to the accuracy or pertinence of the references or that any reference is material to patentability.

What is Claimed is:

1. A method for identifying apoptosis modulating compounds, the method comprising:

(a) contacting a lipid membrane with a pore-forming pro-apoptotic member of the BCL-2 family and a compound to be tested for modulation of apoptotic activity; and

(b) determining whether the compound regulates the formation of large pores in the lipid membrane, where an increase in the number of large pores indicates that the compound is a promoter of apoptitic activity and a decrease in the number of large pores indicates that the compound is an inhibitor of apoptotic activity.

2. The method of claim 1, wherein the lipid membrane has a higher concentration of cytochrome c on one side of the membrane than on the other side and determining whether the compound regulates the formation of large pores in the lipid membrane comprises determining whether the compound regulates the transfer of cytochrome c through the membrane.

3. The method of claim 1, wherein the lipid membrane has a higher concentration of FD10 on one side of the membrane than on the other side and determining whether the compound regulates the formation of large pores in the lipid membrane comprises determining whether the compound regulates the transfer of FD10 through the membrane.

4. The method of claim 1, wherein the pore-forming pro-apoptotic member of the BCL-2 family is Bax.

5. The method of claim 4, wherein the large pores are pores of approximately 20 - 35 Å in size.

6. The method of claim 1, wherein the large pores are pores that are formed by at least four molecules of the pro-apoptotic BCL-2 family member polypeptide.

7. A method of inducing apoptosis in a cell comprising:

(a) selecting a compound which induces the formation of a pore of approximately 20 - 35 Å in a lipid membrane, wherein said selecting comprises testing the compound for the induction of said pore formation; and

(b) administering the compound to the cell.

8. The method of claim 7, wherein the compound is a polypeptide or derivative thereof.

9. The method of claim 8, wherein the polypeptide comprises a BH3 domain of a pro-apoptotic BCL-2 family member or a fragment thereof.

10. The method of claim 9, wherein the pro-apoptotic BCL-2 family member is selected from the group consisting of Bax, Bak, Bcl-x_S, Bad, Bik and Bid.

11. The method of claim 10, wherein the polypeptide comprises SEQ ID NO:1.

12. The method of claim 7, wherein the cell is a cell of a patient with a condition mediated by excessive down-regulation of apoptosis.

13. The method of claim 7, wherein the pore is one that includes at least four molecules of a pro-apoptotic BCL-2 family member.

14. The method of claim 7, wherein the pore is one that releases a compound selected from the group consisting of FD10 and cytochrome c.

15. The method of claim 8, wherein the patient is a human and the condition is selected from the group consisting of neoplasias, diseases caused by Epstein-Barr virus, African swine fever virus, and adenovirus, lymphoproliferative conditions, cancer, arthritis, Crohn's disease, inflammation, and autoimmune disease.

16. A method of inhibiting apoptosis in a cell comprising:

(a) selecting a compound which inhibits the formation of a pore of approximately 20 - 35 Å in a lipid membrane, wherein said selecting comprises testing the compound for the inhibition of said pore formation; and

(b) administering the compound to the cell.

17. The method of claim 16, wherein the compound is a polypeptide or derivative thereof.

18. The method of claim 17, wherein the polypeptide comprises a BH3 domain of an anti-apoptotic BCL-2 family member or a fragment thereof.

19. The method of claim 18, wherein the anti-apoptotic BCL-2 family member is selected from the group consisting of Bcl-2, Bcl-x_L, Bcl-W, Mcl-1, A-1, and NR-13.

20. The method of claim 19, wherein the polypeptide comprises SEQ ID NO:2.

21. The method of claim 16 wherein the cell is a cell of a patient with a condition mediated by excessive apoptosis.

22. The method of claim 16, wherein the pore is one that includes at least four molecules of a pro-apoptotic BCL-2 family member.

23. The method of claim 16, wherein the pore is one that releases a compound selected from the group consisting of FD10 and cytochrome c.

24. The method of claim 16, wherein the patient is a human and the condition is selected from the group consisting of immunodeficiency diseases, senescence, neurodegenerative diseases, ischemic and reperfusion cell death, infertility, and a wound.

25. A method of treating a patient having a condition mediated by excessive down-regulation of apoptosis comprising:

(a) selecting a compound which induces the formation of a pore of approximately 20 - 35 Å, wherein said selecting comprises testing the compound for the induction of said pore formation; and

(b) administering the compound to the patient.

26. The method of claim 25, wherein the compound is a polypeptide or derivative thereof.

27. The method of claim 26, wherein the polypeptide comprises a BH3 domain of a pro-apoptotic BCL-2 family member or a fragment thereof.

28. The method of claim 27 wherein the pro-apoptotic BCL-2 family member is selected from the group consisting of Bax, Bak, Bcl-x_S, Bad, Bik and Bid.

29. The method of claim 28, wherein the polypeptide comprises SEQ ID NO:1.

30. The method of claim 29, wherein the cell is a cell of a patient with a condition mediated by excessive down-regulation of apoptosis.

31. The method of claim 25, wherein the pore is one that includes at least four molecules of a pro-apoptotic BCL-2 family member.

32. The method of claim 25, wherein the pore is one that releases a compound selected from the group consisting of FD10 and cytochrome c.

33. The method of claim 26, wherein the patient is a human and the condition is selected from the group consisting of neoplasias, diseases caused by Epstein-Barr virus, African swine fever virus, and adenovirus, lymphoproliferative conditions, cancer, arthritis, Crohn's disease, inflammation, and qutoimmune disease.

34. A method of treating a patient having a condition mediated by excessive apoptosis comprising:

(a) selecting a compound which inhibits the formation of a pore of approximately 20 - 35 Å, wherein said selecting comprises testing the compound for the inhibition of said pore formation; and

(b) administering the compound to the patient.

35. The method of claim 34, wherein the compound is a polypeptide or derivative thereof.

36. The method of claim 35, wherein the polypeptide comprises a BH3 domain of an anti-apoptotic BCL-2 family member or a fragment thereof.

37. The method of claim 36, wherein the anti-apoptotic BCL-2 family member is selected from the group consisting of Bcl-2, Bcl-x_L, Bcl-W, Mcl-1, A-1, and NR-13.

38. The method of claim 37, wherein the polypeptide comprises SEQ ID NO:2.

39. The method of claim 38, wherein the cell is a cell of a patient with a condition mediated by excessive apoptosis.

40. The method of claim 34, wherein the pore is one that includes at least four molecules of a pro-apoptotic BCL-2 family member.

41. The method of claim 34, wherein the patient is a human and the condition is selected from the group consisting of immunodeficiency diseases, senescence, neurodegenerative diseases, ischemic and reperfusion cell death, infertility, and a wound.

42. A compound that modulates the formation of large pores in a lipid membrane.

43. The compound of claim 42, wherein the large pores are pores that involve at least four molecules of a pro-apoptotic BCL-2 family member polypeptide.

44. The compound of claim 37, wherein the large pores are pores of about 20 - 35 Å in size.

45. The compound of claim 44, wherein the modulation is the promotion of the formation of large pores.

46. The compound of claim 44, wherein the modulation is the inhibition of the formation of large pores.

47. The compound of claim 43, wherein the pro-apoptotic BCL-2 family member polypeptide is Bax.

48. The compound of claim 43, wherein the pro-apoptotic BCL-2 family member polypeptide is one that includes SEQ ID NO:1.

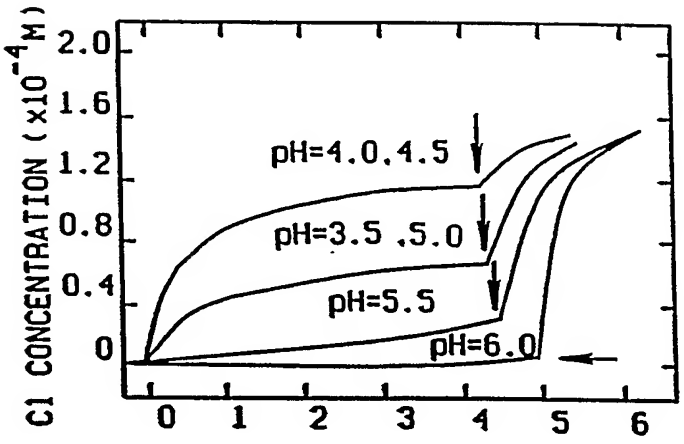


FIGURE 1A

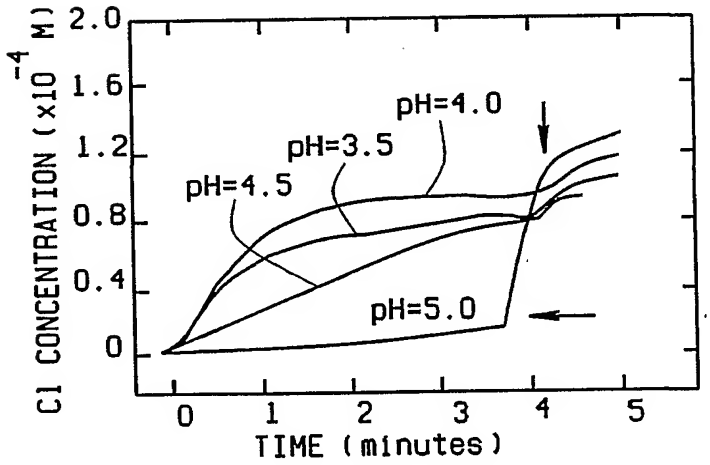


FIGURE 1B

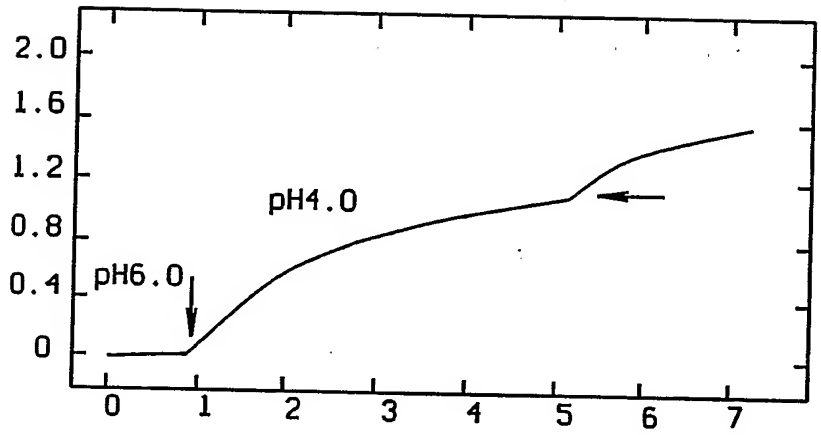


FIGURE 1C

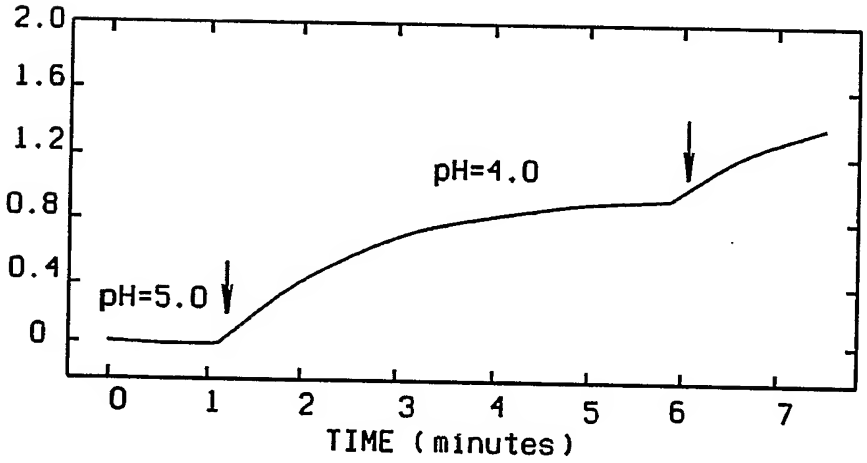


FIGURE 1D

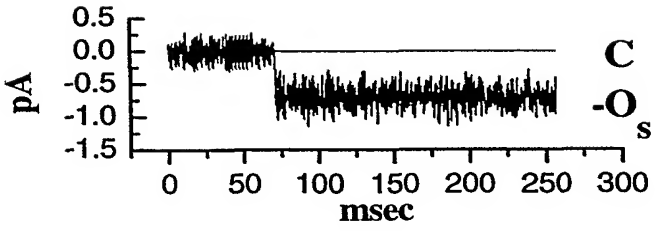


FIGURE 2A

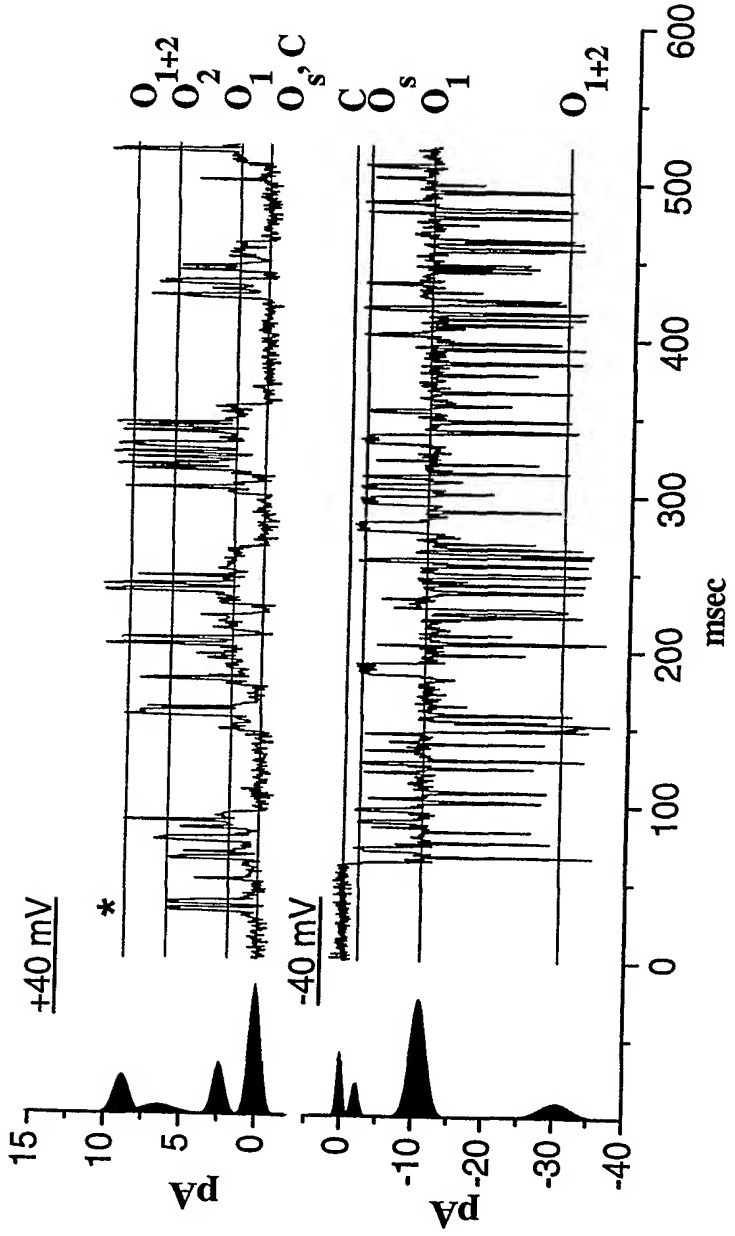

O1+2
O2
O1
Os'
C
C
Os
O1
O1+2
+40 mV
-40 mV
*
pA
pA
msec
15
10
5
0
0
-10
-20
-30
-40
0
100
200
300
400
500
600

FIGURE 2B

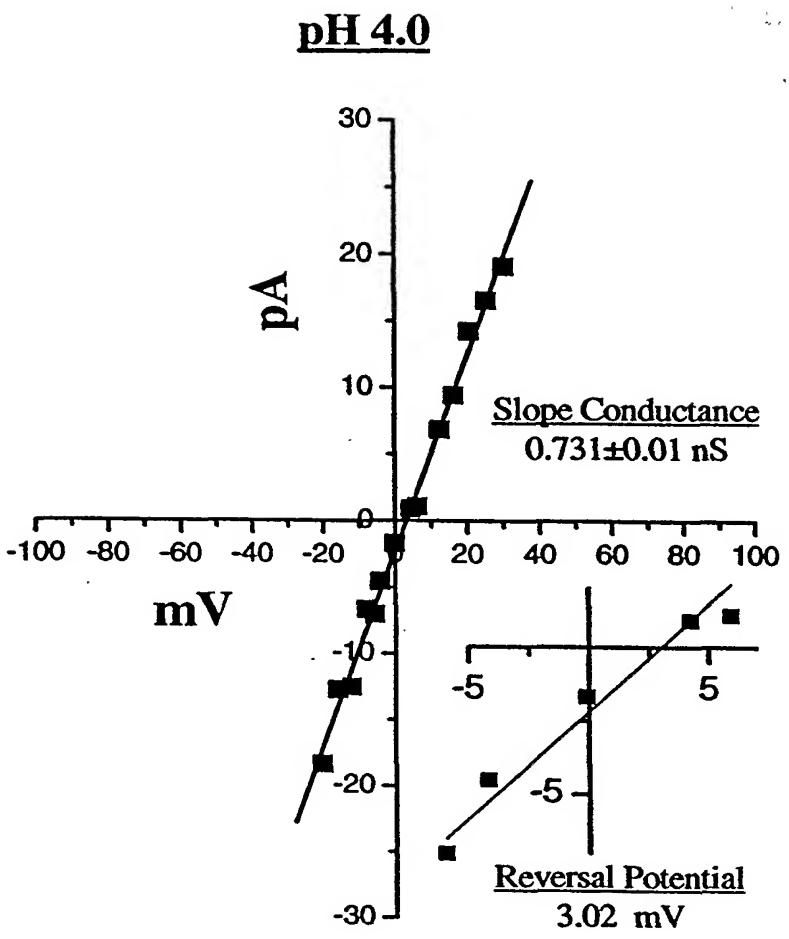

pH 4.0
30
20
10
0
-10
-20
-30
pA
-100 -80 -60 -40 -20 20 40 60 80 100
mV
Slope Conductance
0.731±0.01 nS
-5
5
-5
Reversal Potential
3.02 mV

FIGURE 2C

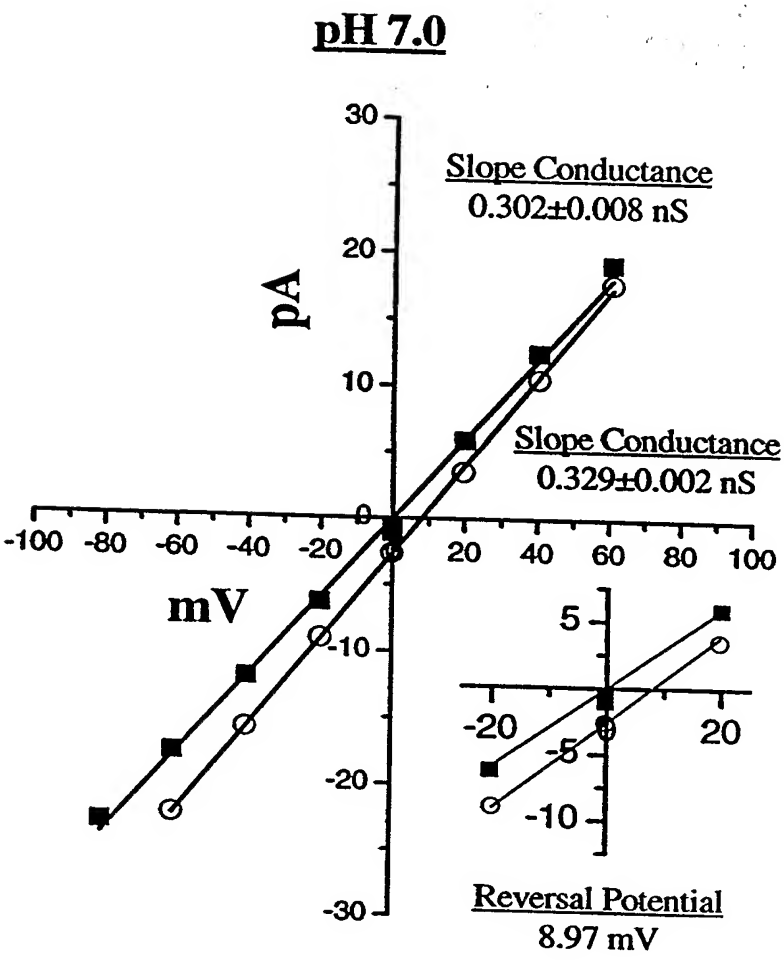

pH 7.0
Slope Conductance
0.302±0.008 nS
Slope Conductance
0.329±0.002 nS
Reversal Potential
8.97 mV
pA
mV
30
20
10
0
-10
-20
-30
-100
-80
-60
-40
-20
20
40
60
80
100
5
-5
-10

FIGURE 2D

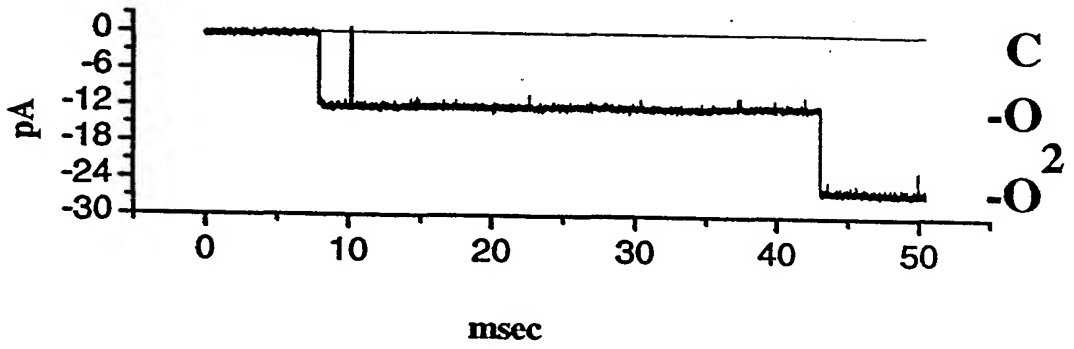


FIGURE 3A

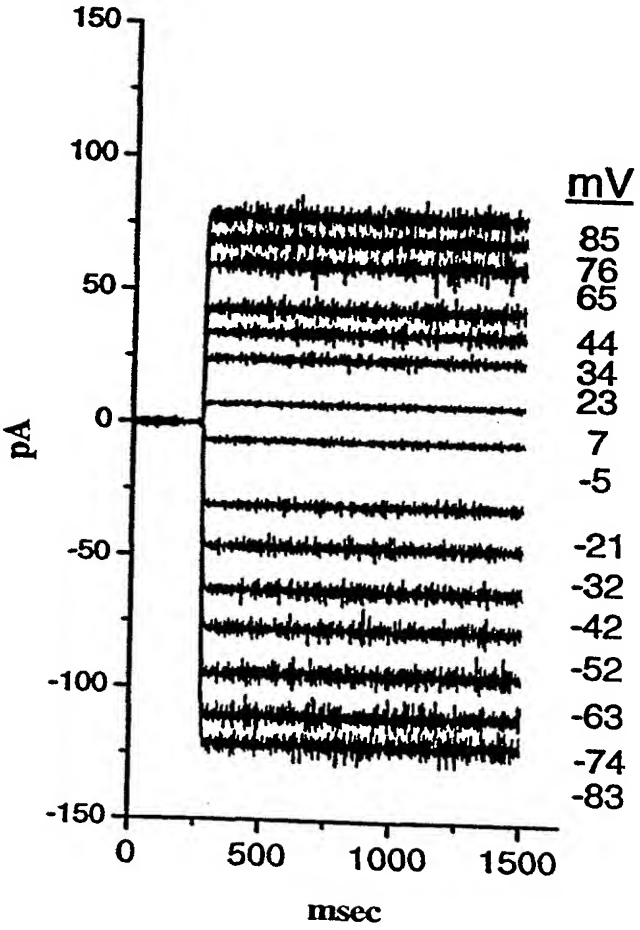


FIGURE 3B

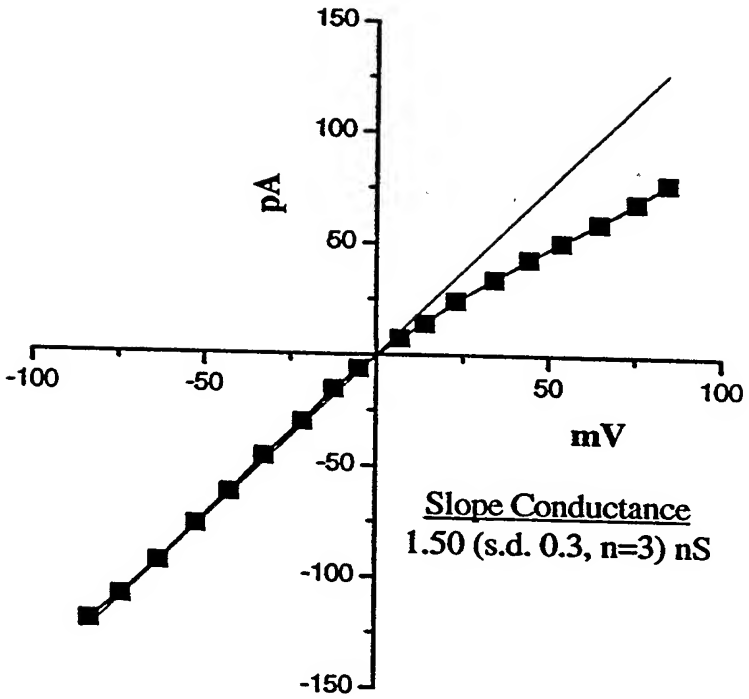


FIGURE 3C

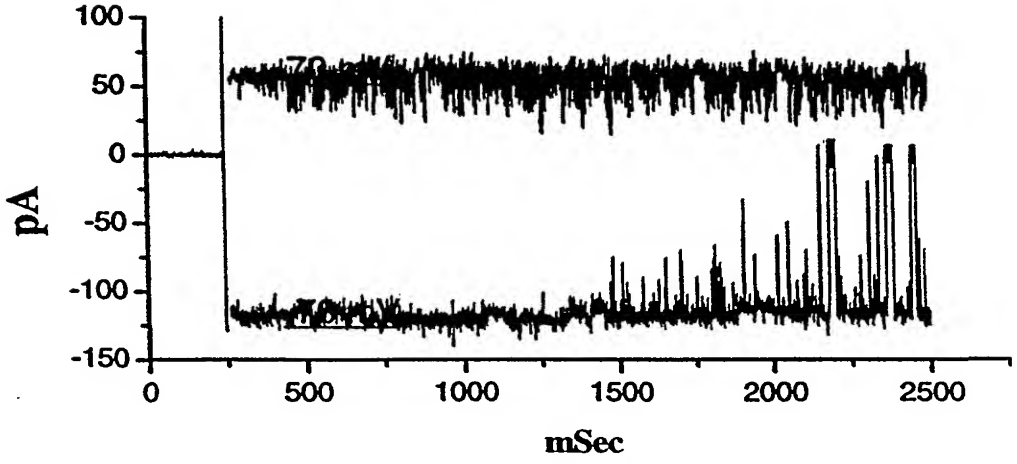


FIGURE 3D

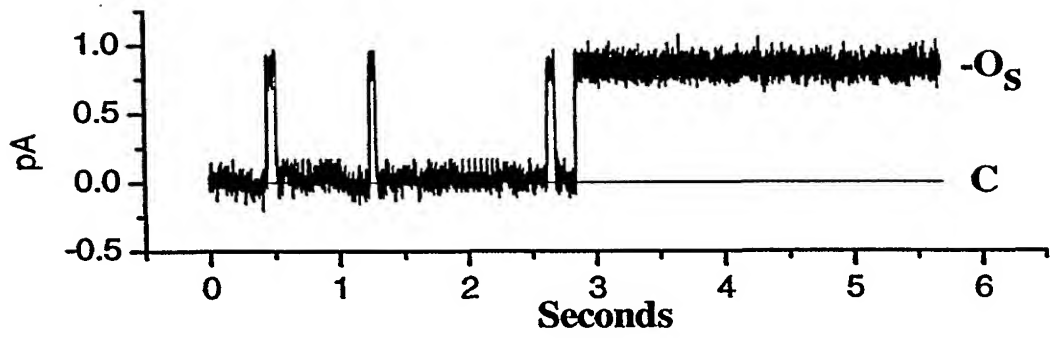


FIGURE 4A

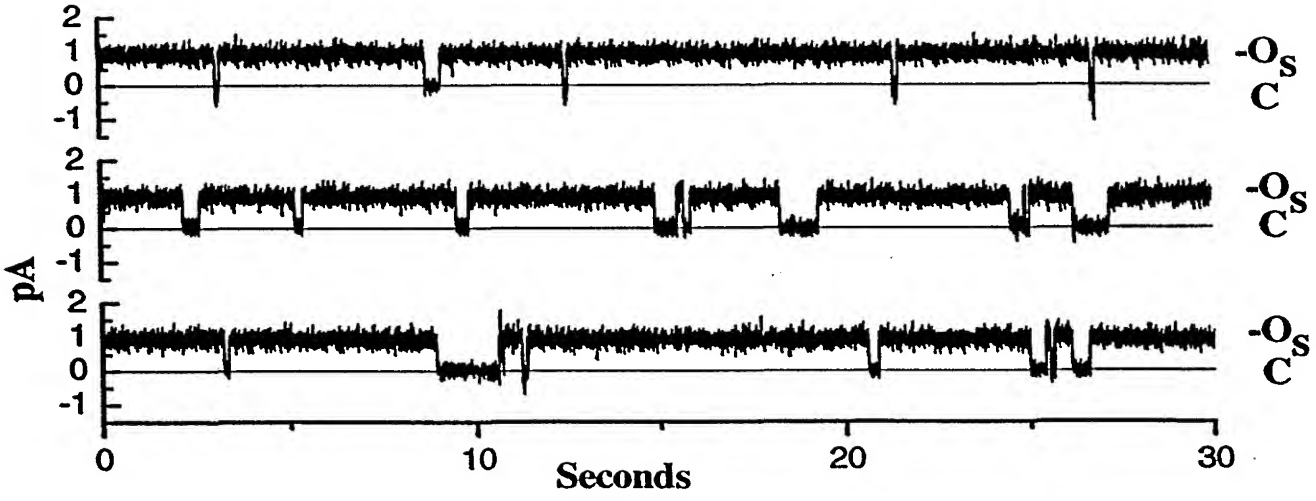


FIGURE 4B

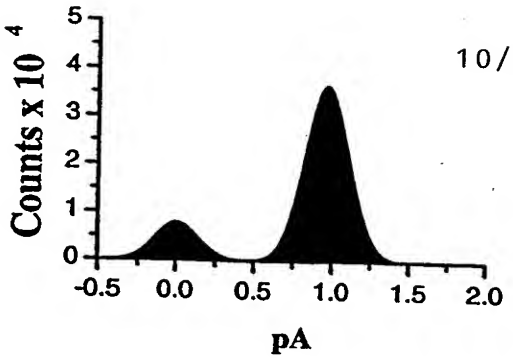


FIGURE 4C

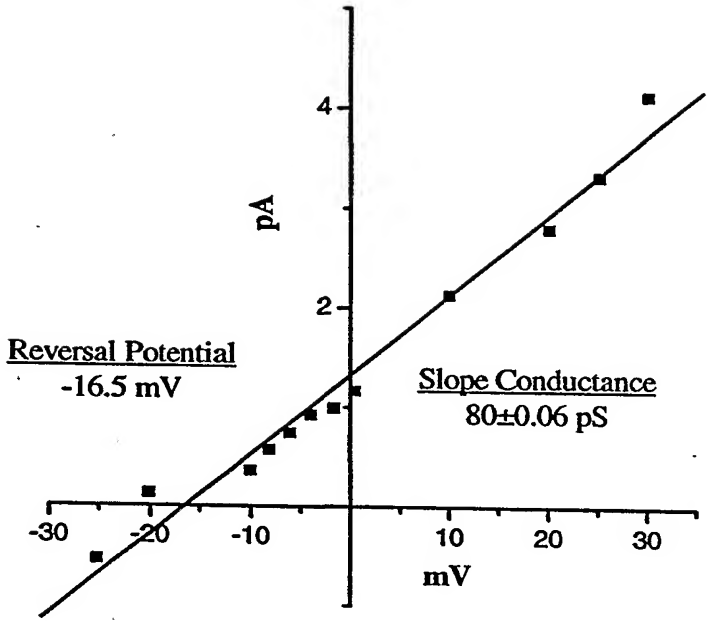


FIGURE 4D

	Conditions	Slope Conductance	Reversal Potential
□	BCL-2 pH 7	2.14±0.04 nS	-19.1 mV
■	BCL-2 pH 4	1.90±0.06 nS	-18.0 mV

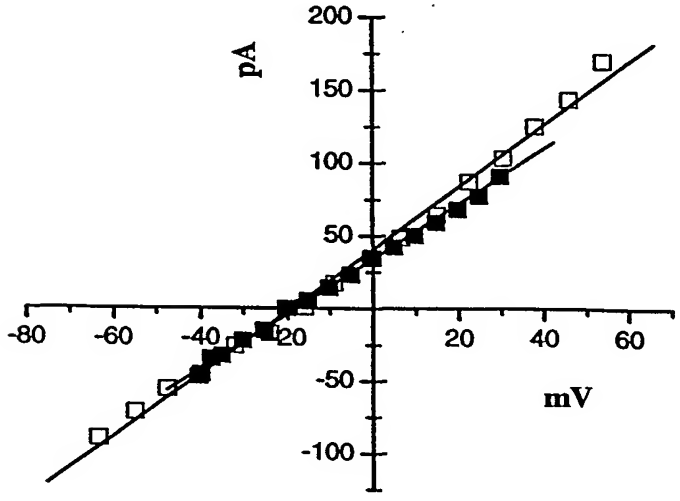


FIGURE 4E

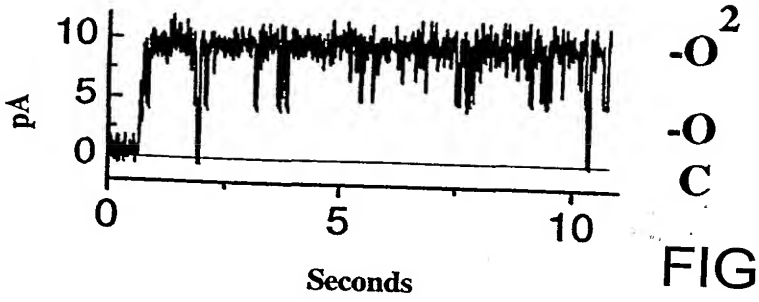


FIGURE 5A

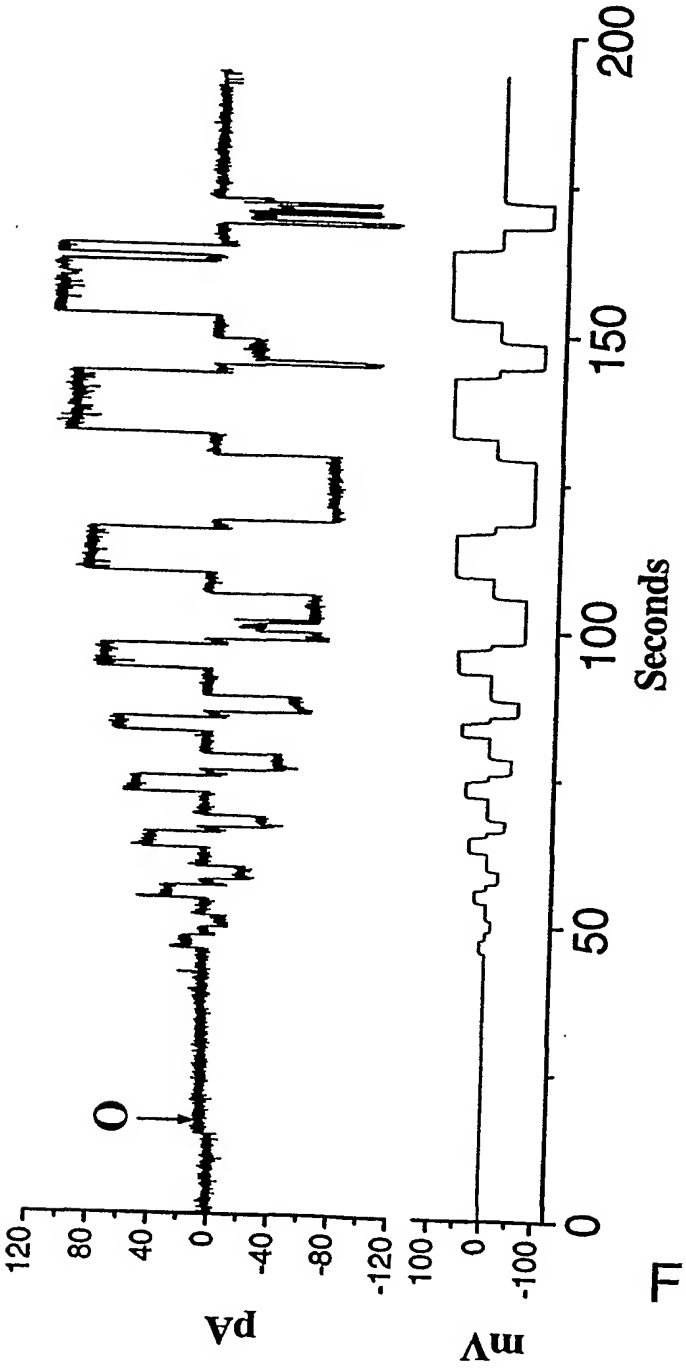


FIGURE 5B

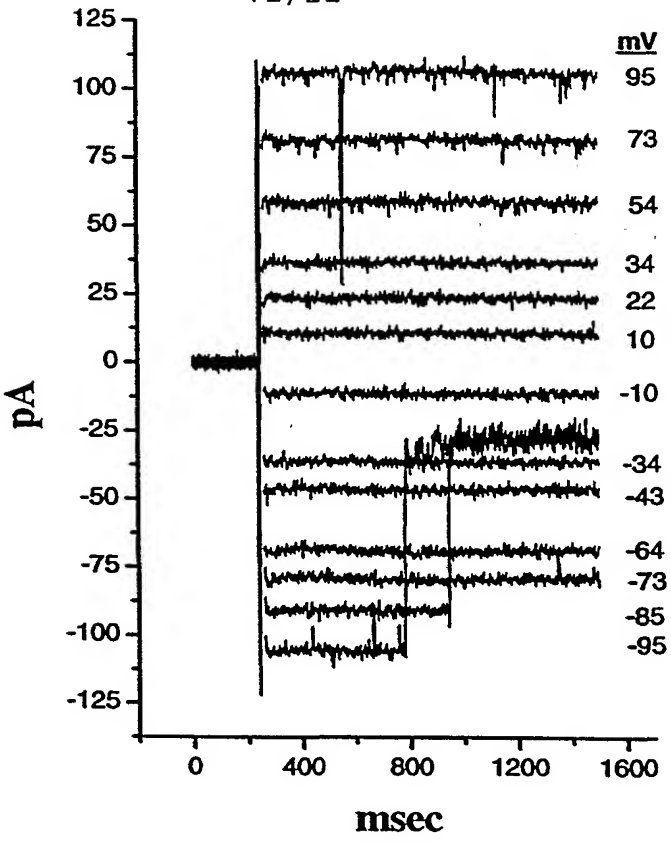


FIGURE 5C

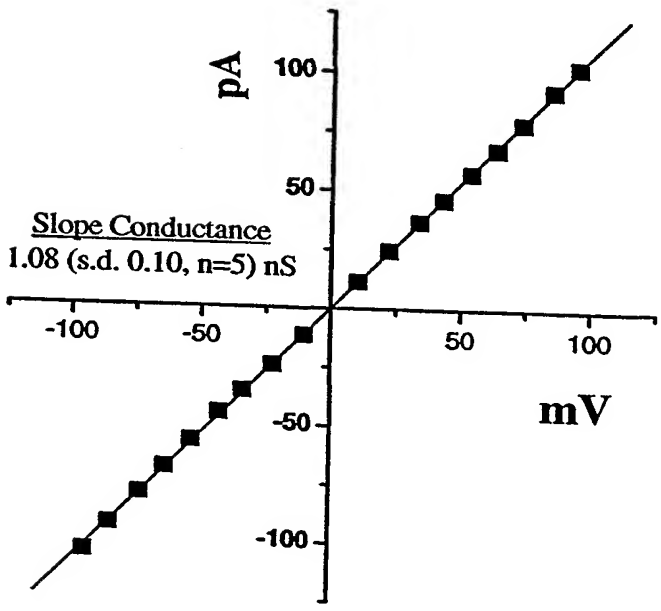


FIGURE 5D

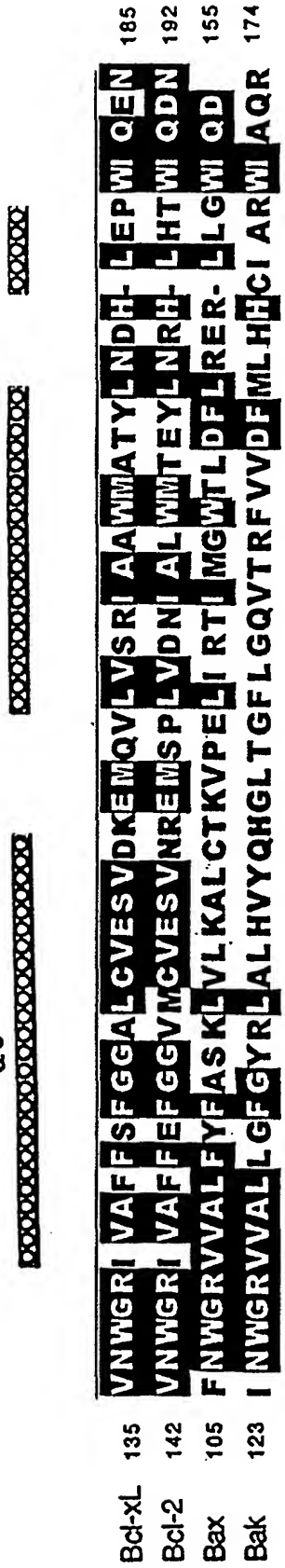

α5
α6
α6
Bcl-xL 135 VNWGRIVAFFSFGGALCVESVDKEMQVLVSRIAAWMATYLNDHLEPWIQEN 185
Bcl-2 142 VNWGRIVAFFEFGGVMCVESVNREMSPLVDNIALWMTEYLNRHLHTWIQDN 192
Bax 105 FNWGRVVALFYFASKLVLKALCTKVPELIRTIMGWTLDFLRERLLGWIQD 155
Bak 123 INWGRVVALLGFGYRLALHVYQHGLTGFLGQVTRFVVDFMLHHCIARWIAQR 174

FIGURE 7

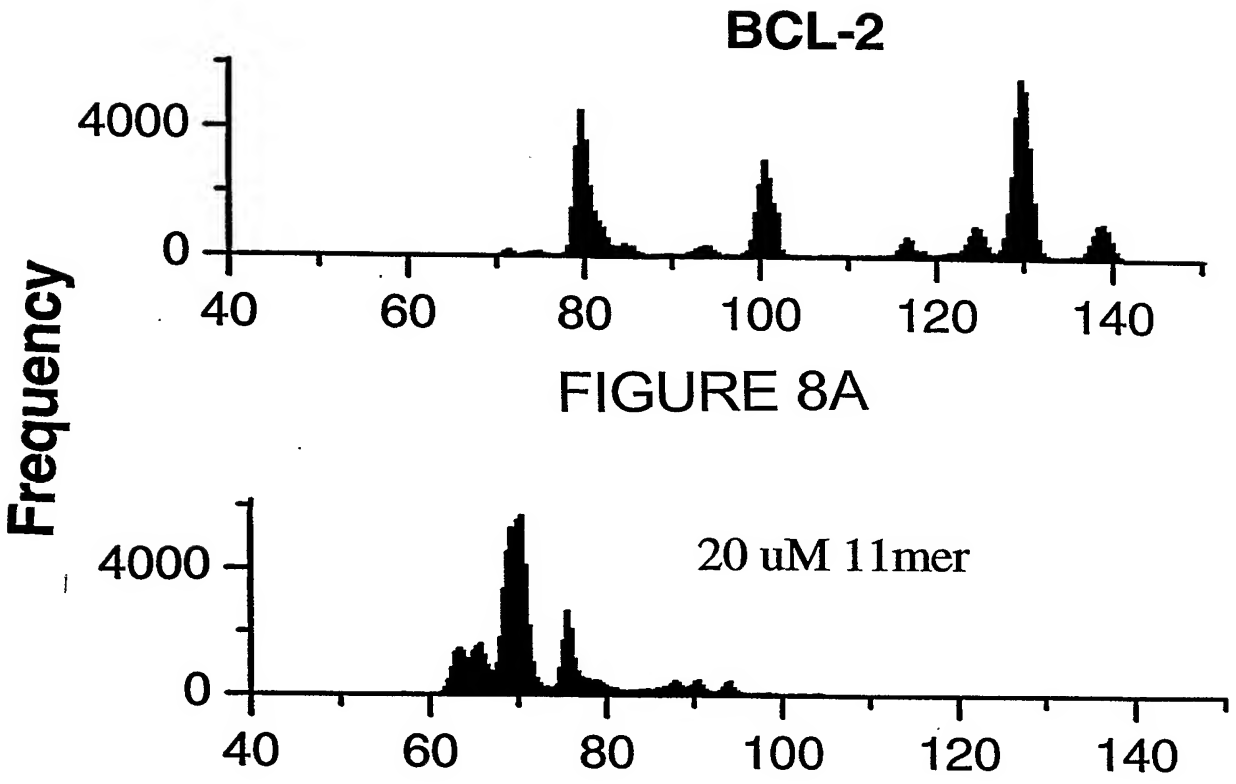


FIGURE 8A

FIGURE 8B

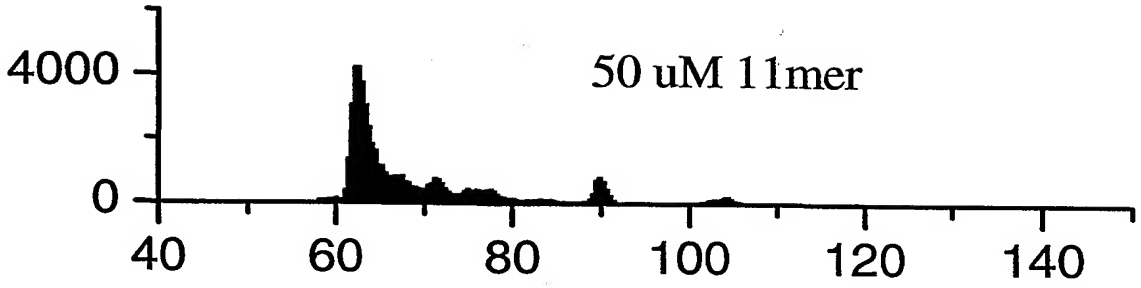


FIGURE 8C

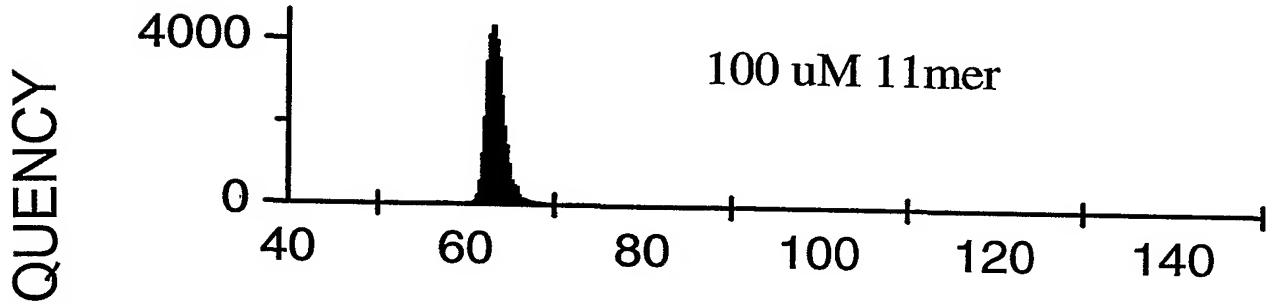


FIGURE 8D

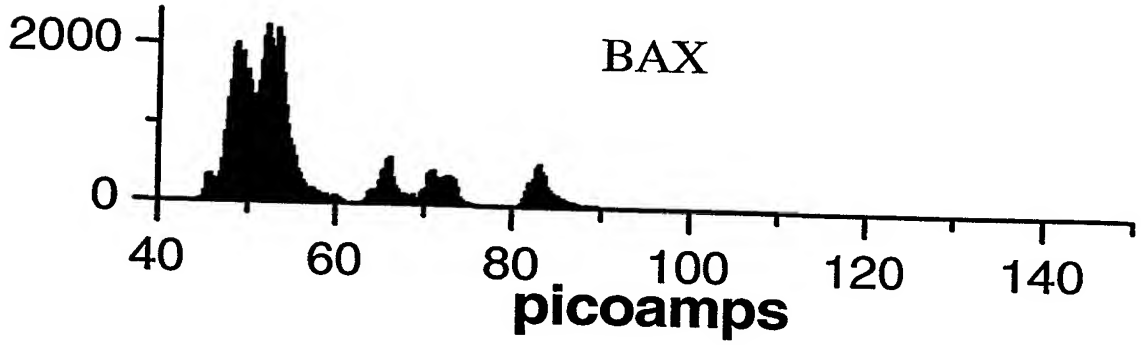
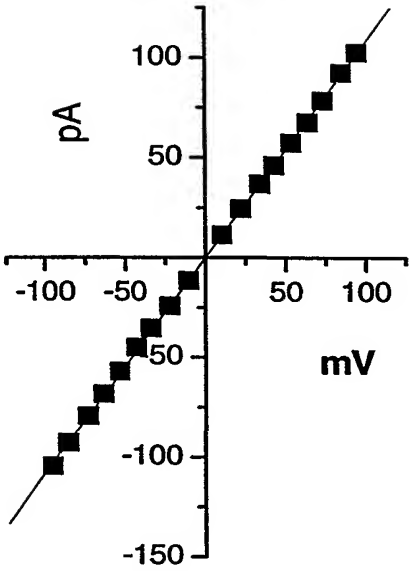


FIGURE 8E

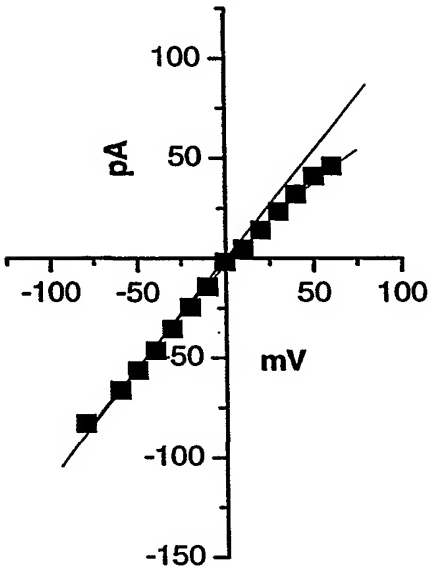
BCL-2



slope = 1.08±0.003 ps
pK/pCl = 2.4

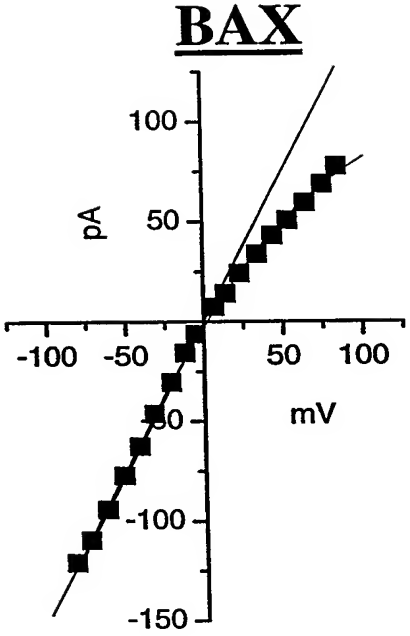
FIGURE 9A

BCL-2 + 150 μM 11mer



slope (neg potentials) =
1.08±0.022 pS
pK/pCl = 0.26±0.05

FIGURE 9B



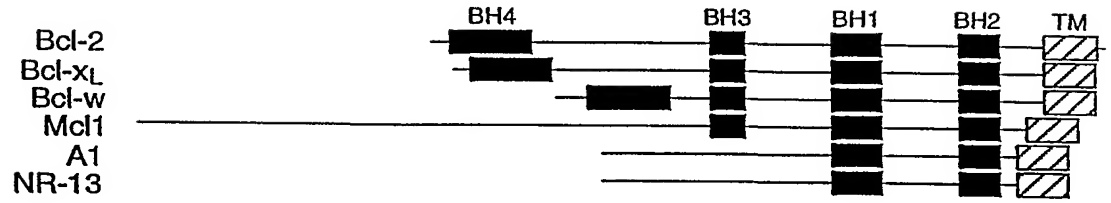
slope(neg potentials) =
1.50±0.001 pS
pK/Pcl = 0.32

FIGURE 9C

THE BCL-2 FAMILY

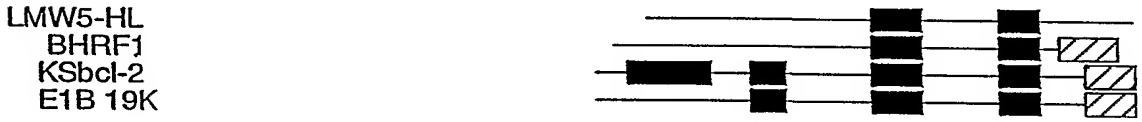
ANTI-APOPTOTIC

MAMMALIAN

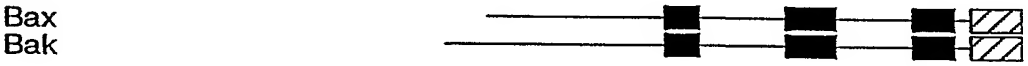


C. elegans

VIRAL HOMOLOGS



PRO-APOPTOTIC



PRO-APOPTOTIC — BH3

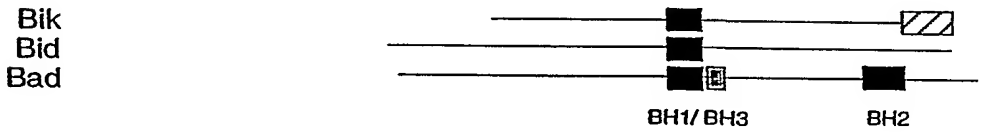


FIGURE 10

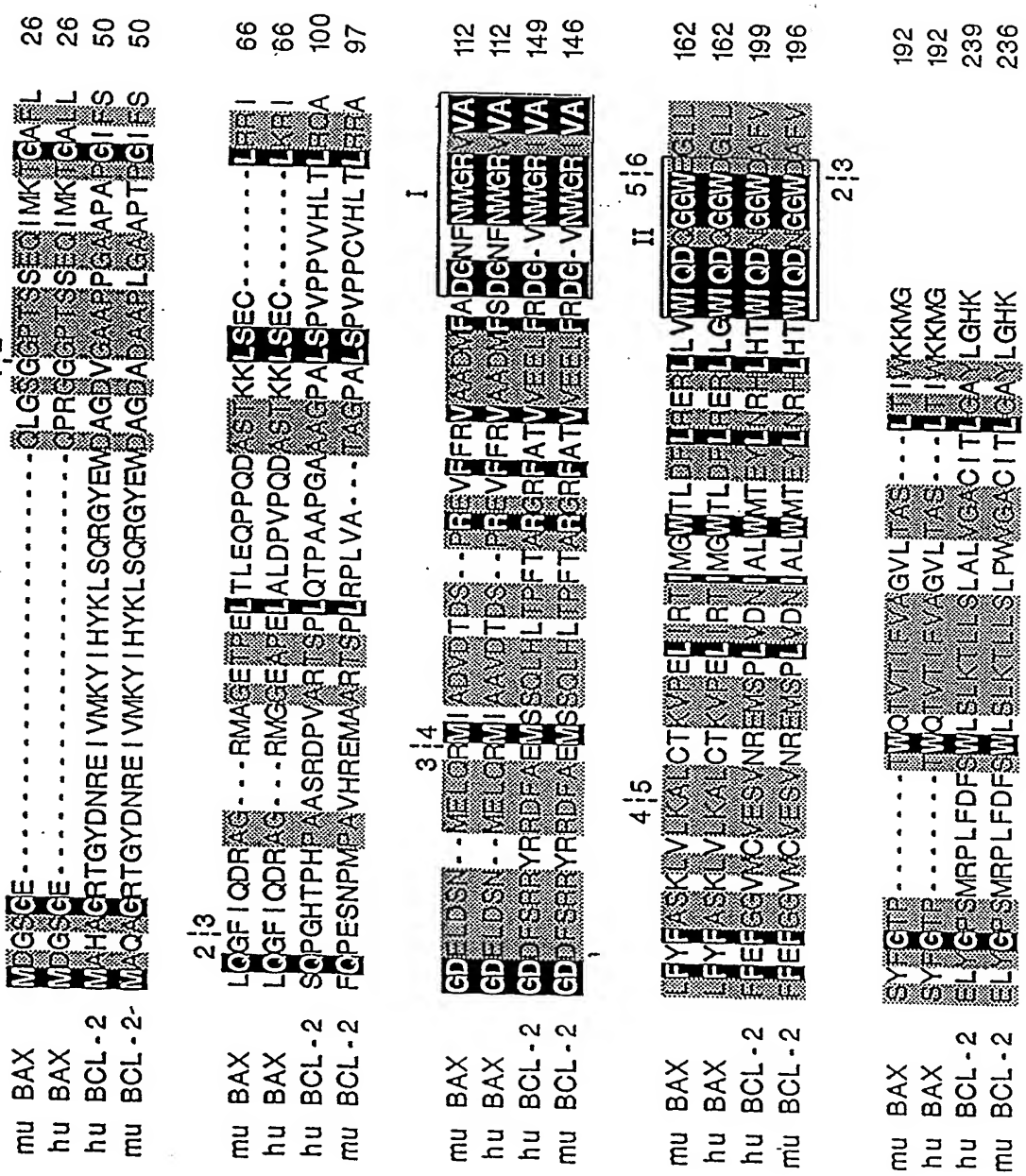


FIGURE 11

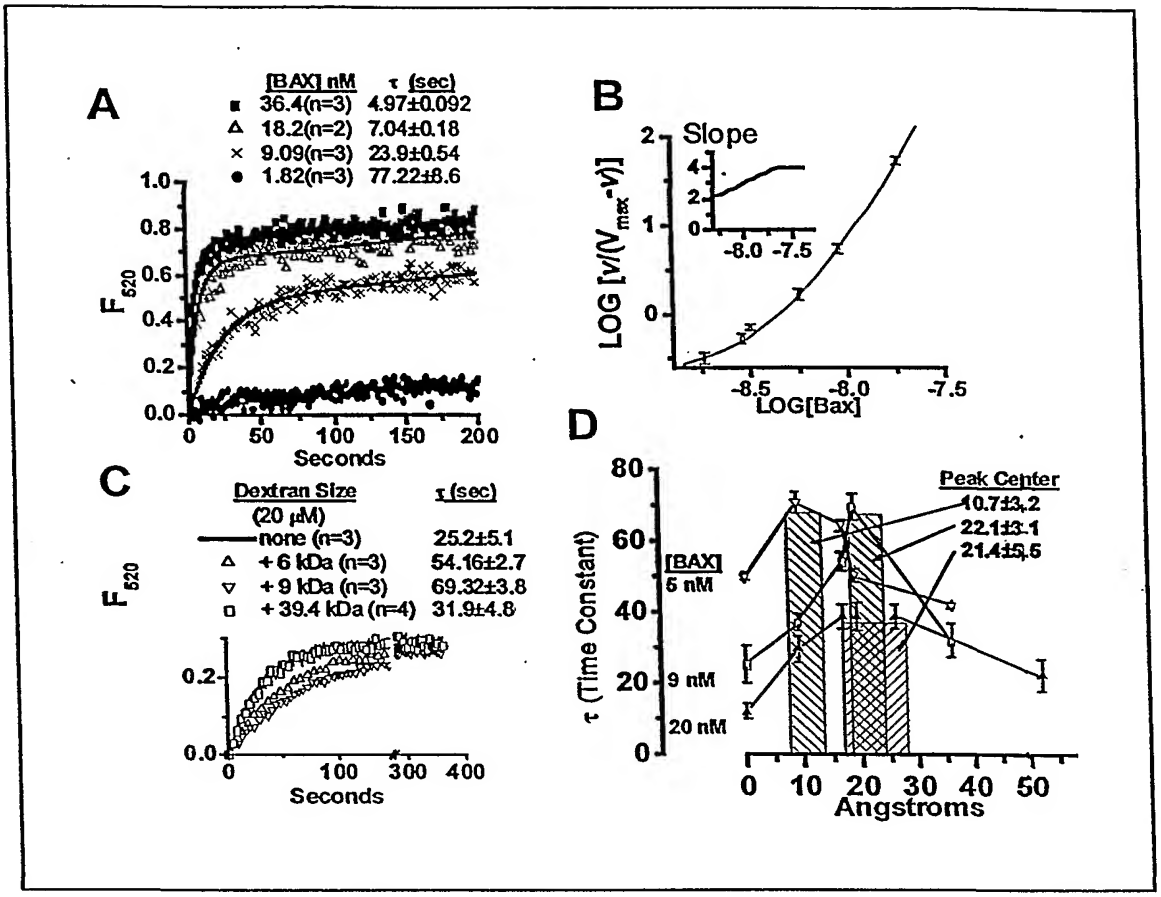

A
[BAX] nM τ (sec)
36.4(n=3) 4.97±0.092
18.2(n=2) 7.04±0.18
9.09(n=3) 23.9±0.54
1.82(n=3) 77.22±8.6
F620
Seconds
B
Slope
LOG [v/(Vmax-v)]
LOG[Bax]
C
Dextran Size (20 μM) τ (sec)
none (n=3) 25.2±5.1
+6 kDa (n=3) 54.16±2.7
+9 kDa (n=3) 69.32±3.8
+39.4 kDa (n=4) 31.9±4.8
F620
Seconds
D
τ (Time Constant)
[BAX]
5 nM
9 nM
20 nM
Peak Center
10.7±3.2
22.1±3.1
21.4±5.6
Angstroms

FIGURE 12

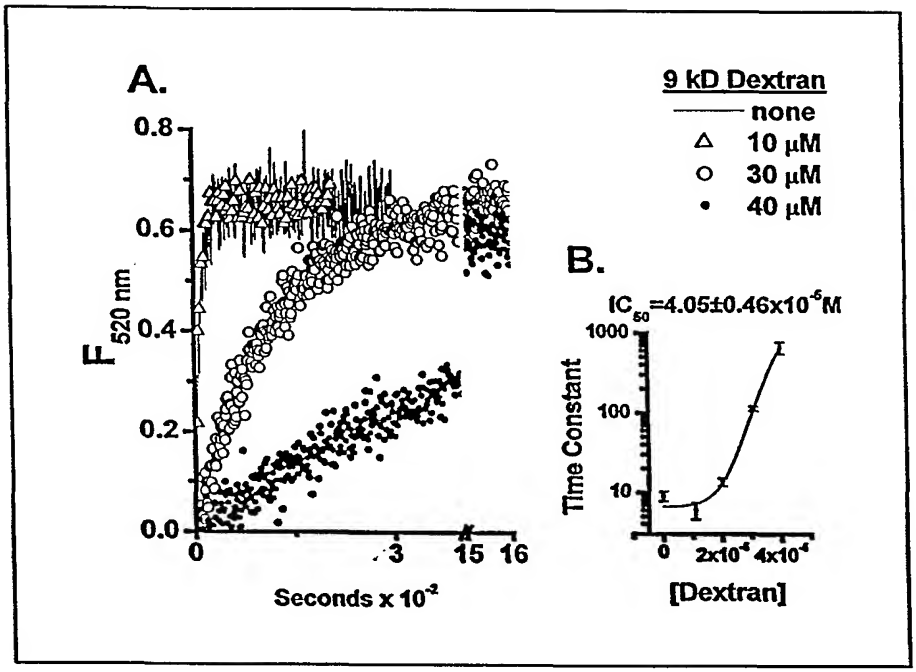

A.
9 kD Dextran
none
10 μM
30 μM
40 μM
0.8
0.6
0.4
0.2
0.0
F 520 nm
0
3
15
16
Seconds x 10⁻²
B.
IC50=4.05±0.46x10⁻⁵M
1000
100
10
Time Constant
0
2x10⁻⁴
4x10⁻⁴
[Dextran]

FIGURE 13

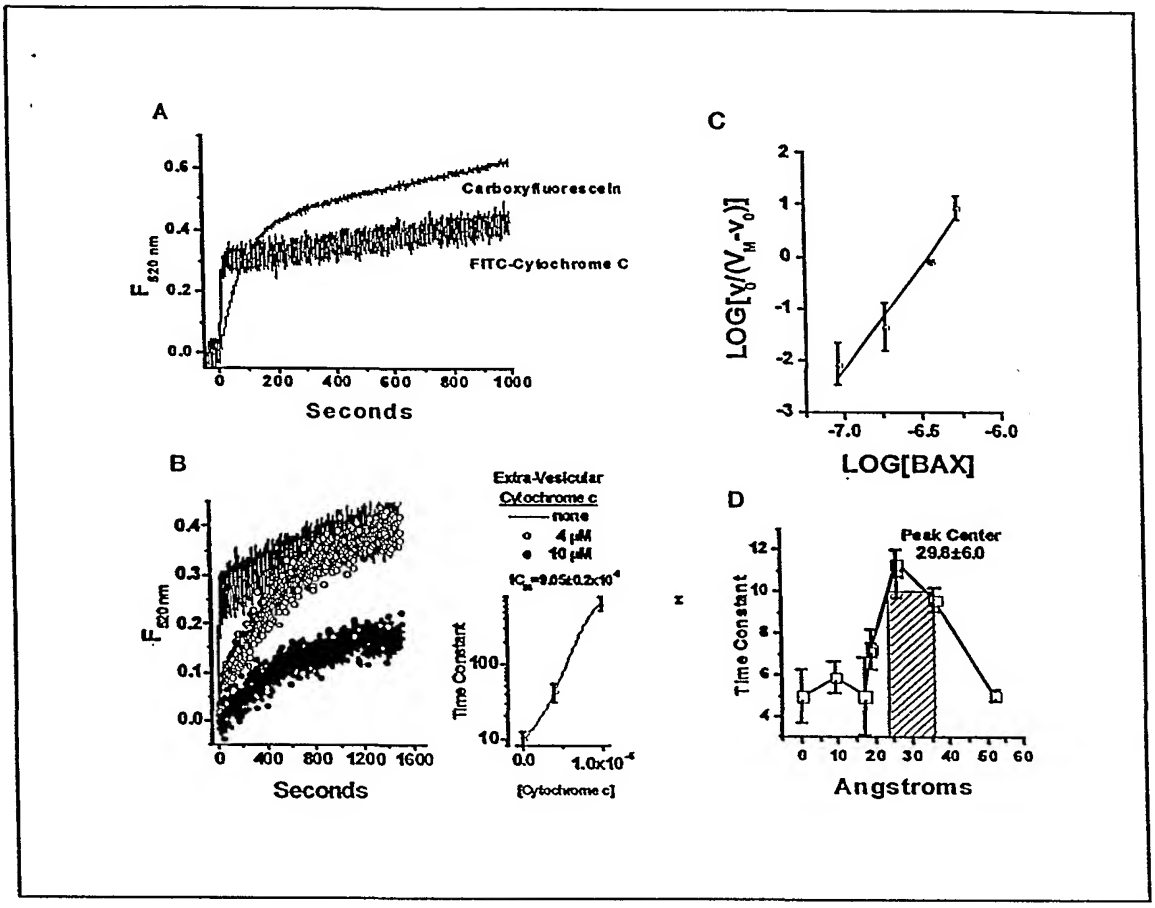

A
Carboxyfluorescein
FITC-Cytochrome C
Seconds
B
Extra-Vesicular
Cytochrome c
none
4 μM
10 μM
Seconds
Time Constant
[Cytochrome c]
C
LOG[BAX]
D
Peak Center
29.8±6.0
Time Constant
Angstroms

FIGURE 14

SEQUENCE LISTING

<110> Korsmeyer, Stanley J.
Schlesinger, Paul H.

<120> Modulation of Apoptosis

<130> 6029-7984

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<150> 09/617,878
<151> 2000-07-17

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<170> PatentIn Ver. 2.0

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1 5 10

<210> 3
<211> 51
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<213> mammalian

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Cys Val Glu Ser Val Asp Lys Glu Met Gln Val Leu Val Ser Arg Ile
20 25 30

Ala Ala Trp Met Ala Thr Tyr Leu Asn Asp His Leu Glu Pro Trp Ile
35 40 45

Gln Glu Asn
50

<210> 4
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<212> PRT
<213> mammalian

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1 5 10 15

Cys Val Glu Ser Val Asn Arg Glu Met Ser Pro Leu Val Asp Asn Ile
20 25 30

Ala Leu Trp Met Thr Glu Tyr Leu Asn Arg His Leu His Thr Trp Ile
35 40 45

Gln Asp Asn
50

<210> 5
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<213> mammalian

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Phe Asn Trp Gly Arg Val Val Ala Leu Phe Tyr Phe Ala Ser Lys Leu
1 5 10 15

Val Lys Leu Ala Leu Cys Thr Lys Val Pro Glu Leu Ile Arg Thr Ile
20 25 30

Met Gly Trp Thr Leu Asp Phe Leu Arg Glu Arg Leu Leu Gly Trp Ile
35 40 45

Gln Asp Gln
50

<210> 6
<211> 52
<212> PRT
<213> mammalian

<400> 6
Ile Asn Trp Gly Arg Val Val Ala Leu Leu Gly Phe Gly Tyr Arg Leu
1 5 10 15

Ala Leu His Val Tyr Gln His Gly Leu Thr Gly Phe Leu Gly Gln Val
20 25 30

Thr Arg Phe Val Val Asp Phe Met Leu His His Cys Ile Ala Arg Trp
35 40 45

Ile Ala Gln Arg
50

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1 5 10 15

Glu Gln Ile Met Lys Thr Gly Ala Phe Leu Leu Gln Gly Phe Ile Gln
20 25 30

Asp Arg Ala Gly Arg Met Ala Gly Glu Thr Pro Glu Leu Thr Leu Glu
35 40 45

Gln Pro Pro Gln Asp Ala Ser Thr Lys Lys Leu Ser Glu Cys Leu Arg
50 55 60

```
Arg Ile Gly Asp Glu Leu Asp Ser Asn Met Glu Leu Gln Arg Met Ile
 65                  70                  75                  80

Ala Asp Val Asp Thr Asp Ser Pro Arg Glu Val Phe Phe Arg Val Ala
                85                  90                  95

Ala Asp Met Phe Ala Asp Gly Asn Phe Asn Trp Gly Arg Val Val Ala
            100                 105                 110

Leu Phe Tyr Phe Ala Ser Lys Leu Val Leu Lys Ala Leu Cys Thr Lys
        115                 120                 125

Val Pro Glu Leu Ile Arg Thr Ile Met Gly Trp Thr Leu Asp Phe Leu
    130                 135                 140

Arg Glu Arg Leu Leu Val Trp Ile Gln Asp Gln Gly Gly Trp Glu Gly
145                 150                 155                 160

Leu Leu Ser Tyr Phe Gly Thr Pro Thr Trp Gln Thr Val Thr Ile Phe
                165                 170                 175

Val Ala Gly Val Leu Thr Ala Ser Leu Thr Ile Trp Lys Lys Met Gly
            180                 185                 190
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<213> Homo sapiens

<400> 8

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Glu Gln Ile Met Lys Thr Gly Ala Leu Leu Leu Gln Gly Phe Ile Gln
             20                  25                  30

Asp Arg Ala Gly Arg Met Gly Gly Glu Ala Pro Glu Leu Ala Leu Asp
         35                  40                  45

Pro Val Pro Gln Asp Ala Ser Thr Lys Lys Leu Ser Glu Cys Leu Lys
     50                  55                  60

Arg Ile Gly Asp Glu Leu Asp Ser Asn Met Glu Leu Gln Arg Met Ile
 65                  70                  75                  80

Ala Ala Val Asp Thr Asp Ser Pro Arg Glu Val Phe Phe Arg Val Ala
                85                  90                  95

Ala Asp Met Phe Ser Asp Gly Asn Phe Asn Trp Gly Arg Val Val Ala
            100                 105                 110

Leu Phe Tyr Phe Ala Ser Lys Leu Val Leu Lys Ala Leu Cys Thr Lys
        115                 120                 125

Val Pro Glu Leu Ile Arg Thr Ile Met Gly Trp Thr Leu Asp Phe Leu
    130                 135                 140

Arg Glu Arg Leu Leu Gly Trp Ile Gln Asp Gln Gly Gly Trp Asp Gly
145                 150                 155                 160
```

```
Leu Leu Ser Tyr Phe Gly Thr Pro Thr Trp Gln Thr Val Thr Ile Phe
                165                 170                 175

Val Ala Gly Val Leu Thr Ala Ser Leu Thr Ile Trp Lys Lys Met Gly
            180                 185                 190
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<210> 9
<211> 239
<212> PRT
<213> Homo sapiens

<400> 9

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Met Ala His Ala Gly Arg Thr Gly Tyr Asp Asn Arg Glu Ile Val Met
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Lys Tyr Ile His Tyr Lys Leu Ser Gln Arg Gly Tyr Glu Trp Asp Ala
            20                  25                  30

Gly Asp Val Gly Ala Ala Pro Pro Gly Ala Ala Pro Ala Pro Gly Ile
        35                  40                  45

Phe Ser Ser Gln Pro Gly His Thr Pro His Pro Ala Ala Ser Arg Asp
    50                  55                  60

Pro Val Ala Arg Thr Ser Pro Leu Gln Thr Pro Ala Ala Pro Gly Ala
65                  70                  75                  80

Ala Ala Gly Pro Ala Leu Ser Pro Val Pro Pro Val Val His Leu Thr
                85                  90                  95

Leu Arg Gln Ala Gly Asp Asp Phe Ser Arg Arg Tyr Arg Arg Asp Phe
            100                 105                 110

Ala Glu Met Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly
        115                 120                 125

Arg Phe Ala Thr Val Val Glu Glu Leu Phe Arg Asp Gly Val Asn Trp
    130                 135                 140

Gly Arg Ile Val Ala Phe Phe Glu Phe Gly Gly Val Met Cys Val Glu
145                 150                 155                 160

Ser Val Asn Arg Glu Met Ser Pro Leu Val Asp Asn Ile Ala Leu Trp
                165                 170                 175

Met Thr Glu Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn
            180                 185                 190

Gly Gly Trp Asp Ala Phe Val Glu Leu Tyr Gly Pro Ser Met Arg Pro
        195                 200                 205

Leu Phe Asp Phe Ser Trp Leu Ser Leu Lys Thr Leu Leu Ser Leu Ala
    210                 215                 220

Leu Val Gly Ala Cys Ile Thr Leu Gly Ala Tyr Leu Gly His Lys
225                 230                 235
```

<210> 10
<211> 236
<212> PRT
<213> Mouse

<400> 10

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Met Ala Gln Ala Gly Arg Thr Gly Tyr Asp Asn Arg Glu Ile Val Met
  1               5                  10                  15

Lys Tyr Ile His Tyr Lys Leu Ser Gln Arg Gly Tyr Glu Trp Asp Ala
            20                  25                  30

Gly Asp Ala Asp Ala Ala Pro Leu Gly Ala Ala Pro Thr Pro Gly Ile
        35                  40                  45

Phe Ser Phe Gln Pro Glu Ser Asn Pro Met Pro Ala Val His Arg Glu
    50                  55                  60

Met Ala Ala Arg Thr Ser Pro Leu Arg Pro Leu Val Ala Thr Ala Gly
65                  70                  75                  80

Pro Ala Leu Ser Pro Val Pro Pro Cys Val His Leu Thr Leu Arg Arg
                85                  90                  95

Ala Gly Asp Asp Phe Ser Arg Arg Tyr Arg Arg Asp Phe Ala Glu Met
            100                 105                 110

Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly Arg Phe Ala
        115                 120                 125

Thr Val Val Glu Glu Leu Phe Arg Asp Gly Val Asn Trp Gly Arg Ile
    130                 135                 140

Val Ala Phe Phe Glu Phe Gly Gly Val Met Cys Val Glu Ser Val Asn
145                 150                 155                 160

Arg Glu Met Ser Pro Leu Val Asp Asn Ile Ala Leu Trp Met Thr Glu
                165                 170                 175

Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn Gly Gly Trp
            180                 185                 190

Asp Ala Phe Val Glu Leu Tyr Gly Pro Ser Met Arg Pro Leu Phe Asp
        195                 200                 205

Phe Ser Trp Leu Ser Leu Lys Thr Leu Leu Ser Leu Ala Leu Val Gly
    210                 215                 220

Ala Cys Ile Thr Leu Gly Ala Tyr Leu Gly His Lys
225                 230                 235
```